Promoting training adaptation in human skeletal muscle by blood flow restriction and cold-water immersion

With special emphasis on K^+ regulation and Na^+,K^+-ATPase abundance in different fibre types

Doctor of Philosophy

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June 2018

Abstract

The molecular signals underlying improvements in the skeletal muscle capacity for K^+ regulation and Na^+,K^+-ATPase expression in humans are poorly understood. Further, fibre-type-specific regulation of Na^+,K^+-ATPase isoforms by exercise training appears inadequately explored. This thesis investigated in humans possible mechanisms regulating the muscle's capacity for K^+ regulation and Na^+,K^+-ATPase-isoform expression in different fibre types with exercise training and presents a novel method for fibre type identification of single muscle fibres. Molecular signals (oxidative stress, hypoxia, lactate, AMPK- and Ca^{2+}-signalling) were modulated by exercising with and without blood flow restriction (BFR), and in systemic hypoxia, and changes in expression of Na^+,K^+-ATPase genes were examined by RT-PCR. In another experiment, an intra-subject design was used, where one leg trained with and the other leg without BFR, along with measurement of thigh K^+ release. Effects of cold-water immersion on training-induced adaptations in Na^+,K^+-ATPase isoforms were also examined. The reliability and validity of dot blotting for fibre-type determination of single muscle fibres were evaluated by use of western blotting. Key findings were that increased oxidative stress, AMPK signalling, and disturbance of ionic and redox homeostasis are positively associated with training-induced increases in the capacity for K^+ regulation and Na^+,K^+-ATPase-isoform expression. In contrast, the level of hypoxia and lactate concentration, and modulation of CaMKII signalling, was not related to the regulation of Na^+,K^+-ATPase-isoform expression. Fibre type-dependent adaptations of Na^+,K^+-ATPase expression were associated with improvements in K^+ regulation and exercise tolerance. In addition, dot blotting was valid and reliable for fibre type determination of single muscle fibres. In conclusion, this thesis has identified key mechanisms underlying, and a novel strategy (BFR training) to augment, training-induced improvements in K^+ regulation by human skeletal muscle and presents a valid and reliable method for easy and rapid fibre type determination of individual muscle fibres.

Extended abstract

Aims

The skeletal muscle's capacity for potassium ion (K^+) regulation is essential for physical performance and disease prevention. This capacity is primarily limited by the function of the membrane-bound ion transport pump, the Na^+,K^+-ATPase, which is composed of several subunits (α, β and FXYD) that are expressed in a fibre type-dependent fashion and as different isoforms in muscle (α_{1-3}, β_{1-3}, and FXYD1) that have different functional properties. In humans, the cellular mechanisms regulating the skeletal muscle's capacity for K^+ regulation and Na^+,K^+-ATPase expression are poorly understood. Evidence from animal and cell culture studies has suggested that this regulation may be mediated by perturbations in the concentrations of ions, oxygen, free radicals, and metabolic by-products, as well as signalling transduction through 5'AMP-activated protein kinase (AMPK) and calcium-calmodulin-dependent protein kinase (CaMK). These cellular stressors can be manipulated during exercise by decreasing muscle blood perfusion (blood flow restriction) or arterial oxygen saturation (systemic hypoxia), or by cooling of the tissue (e.g. by immersing the exercised limbs into cold water; cold-water immersion). Thus, these exercise models were used in the present thesis to investigate in humans 1) possible cellular mechanisms regulating the expression of Na^+,K^+-ATPase isoforms and the capacity for K^+ handling in skeletal muscle by exercise training, and 2) how expression of Na^+,K^+-ATPase isoforms is regulated in different muscle fibre types by different types of training. Whilst the physiological significance of undertaking fibre type-specific protein analyses is well-documented, this is presently a laborious and time-consuming procedure. Thus, a third aim was to develop a novel method for easy and rapid fibre type identification of individual fibre segments from a human skeletal muscle sample.

Methods

In paper I, eight healthy men (mean ± SD: 26 ± 5 y and 57.4 ± 6.3 $mL \cdot kg^{-1} \cdot min^{-1}$) completed, in a randomised, crossover fashion, three sessions of aerobic interval exercise without (CON) or with blood flow restriction (BFR), or in systemic hypoxia (HYP, ~3250 m). A muscle sample was collected before (Pre) and after exercise (+0h, +3h) to quantify Na^+,K^+-ATPase mRNA, indicators of oxidative stress (HSP27 protein in type I and II fibres, and *catalase* and *HSP70* mRNA), metabolites, and α-AMPK Thr^{172}/α-AMPK, ACC Ser^{221}/ACC, CaMKII Thr^{287}/CaMKII, and PLBSer^{16}/PLB ratios in type I and II fibres. In paper II, ten recreationally-active men (25 ± 4 y, 49.7 ± 5.3 $mL \cdot kg^{-1} \cdot min^{-1}$) performed six weeks of aerobic interval training with one leg training without (CON-leg) and the other leg with BFR (BFR-leg, pressure: 178 mmHg). Pre and post training, catheters were inserted into the femoral artery and vein, and blood flow was assessed during single-leg, isolated knee-extensor exercise at 25% (Ex1) and 90% of maximal leg aerobic power (Ex2) with intravenous infusion of N-

acetylcysteine (NAC) or saline (placebo), and a resting muscle biopsy was collected to determine the expression of Na^+,K^+-ATPase isoforms in type I and II fibres. In paper III, nineteen recreationally-active men (24±6 y, 79.5±10.8 kg, 44.6±5.8 $mL \cdot kg^{-1} \cdot min^{-1}$) were randomly allocated to complete six weeks of sprint-interval exercise either without (passive rest; CON) or with training sessions followed by cold-water immersion (15 min at 10°C; COLD). Muscle biopsies were obtained before and after training to determine expression of Na^+,K^+-ATPase α_{1-3}, β_{1-3}, and FXYD1, and after recovery treatments (+0 and +3 hours) on the first day of training to measure corresponding mRNA levels. In paper IV, a novel method for fibre type determination of single muscle fibres predicated on dot blotting was validated. Segments of individual fibres were collected, and one-tenth of each segment was dot blotted for the detection of MHCI, IIa, and IIx protein. For validation, fibre types of samples were also determined by western blotting. According to the fibre type identified, segments were pooled to form samples of type I and IIa fibres for western blotting. Further, reproducibility was determined for proteins of different sizes (SERCA1, SERCA2a, CSQ1, CSQ2, Actin, and AMPKβ2) and variability associated with the pooling of a different number of fibres was also evaluated.

Results:

In paper I, muscle hypoxia (deoxygenation measured by near-infrared spectroscopy) was matched between BFR and HYP, and both were higher than CON (~90% vs. ~70% deoxygenation; $p<0.05$). The mRNA level of *FXYD1* increased in BFR only ($p<0.05$) and was associated with increases in indicators of oxidative stress and type-I fibre ACC Ser^{221}/ACC ratio, but dissociated from muscle hypoxia, lactate, and CaMKII signalling. In paper II, performance during exhaustive exercise increased to a greater extent after training in BFR-leg (23%) compared to CON-leg (12%, $p<0.05$), and thigh K^+ release during Ex2 was attenuated in BFR-leg only ($p<0.05$). Before training, NAC depressed K^+ release during Ex1 ($p<0.05$), but not during Ex2 ($p>0.05$). After training, this depressive effect was blunted in BFR-leg ($p<0.05$), whilst the abundance of Na^+,K^+-ATPase-isoform α_1 in type-II (51%), β_1 in type-I (33%), and FXYD1 in type-I (108%) and type-II (60%) fibres was higher in BFR-leg ($p<0.05$; vs. CON-leg). In paper III, sprint-interval training increased ($p<0.05$) the abundance of α_1 and β_3 in both fiber types, β_1 in type-II fibers, and decreased FXYD1 in type-I fibers, whereas α_2 and α_3 abundance was not altered by training ($p>0.05$). COLD after each session did not influence responses to training ($p>0.05$). However, α_2 mRNA increased after the first session in COLD (+0h, $p<0.05$), but not in CON ($p>0.05$). In both conditions, α_1 and β_3 mRNA increased (+3h; $p<0.05$), β_2 mRNA decreased (+3h; $p<0.05$), whereas α_3, β_1, and FXYD1 mRNA remained unchanged ($p>0.05$) after the first session. In paper IV, Identification of MHCI, IIa, IIx and I/IIa-hybrid fibres with dot blotting was confirmed with western blotting. Dot blotting required very little sample (~2 to 10 mg w.w. tissue), was simple to perform, and reduced the time (10 fold) and costs (40-fold) associated with fibre type-specific analysis. In mathematical probability simulations of fibre pooling,

improvements in accuracy of the mean to replicate an entire muscle sample became significantly smaller ($p < 0.05$) after 3 to 9 fibres were added to the pooled sample, depending on the protein being analysed.

Conclusions:

In summary, increased oxidative stress, AMPK signalling, and perturbations in ion and redox homeostasis, during training sessions appear to be important regulators of training-induced increases in the skeletal muscle abundance of Na^+,K^+-ATPase isoforms and its capacity for K^+ regulation in humans. In contrast, the muscle levels of hypoxia and lactate, and modulation of CaMKII signalling, do not appear to play a significant role in training-induced regulation of Na^+,K^+-ATPase-isoform expression. Some Na^+,K^+-ATPase isoforms (α_1 and β_3) are similarly regulated in type I and II fibres by training, whereas others may respond in a fibre-type-specific manner to certain types of training (α_2, β_1, and FXYD1), and this may depend on both training intensity and volume. Adaptations in Na^+,K^+-ATPase isoforms at the fibre-type level with BFR-training were associated with a reduced thigh K^+ release and increased performance during intense exercise, emphasising that changes in abundance of these isoforms at the fibre-type level could be of significant relevance for the skeletal muscle's capacity for K^+ handling and exercise performance in humans. Furthermore, the present results suggest that blood-flow-restricted training is a potent strategy to augment improvements in performance and K^+ regulation during intense exercise in humans. On the other hand, regular use of cold-water immersion after training sessions neither impaired nor improved adaptations in Na^+,K^+-ATPase-isoform abundance to intense training in humans. In addition, dot blotting can be validly and reliably used for qualitative determination of the fibre type of individual fibres dissected from a human skeletal muscle sample. This new method saves considerable time and money, because no gels or transfer step are needed. Dot blotting for fibre type identification clearly creates possibilities for improvements in our understanding of how muscle fibre type may play a crucial regulatory role in skeletal muscle physiology.

Student declaration

I, Danny Christiansen, declare that the PhD by Publication entitled "Promoting training adaptation in human skeletal muscle by blood flow restriction and cold-water immersion - With special emphasis on K+ regulation and Na^+,K^+-ATPase abundance in different fibre types" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature: 

Date: 22/6-2018.

Acknowledgements

I wish to express my deepest gratitude and appreciation to Prof. David Bishop for excellent supervision, especially during my first years in Australia, and for providing invaluable advice on project design, scientific writing and oral presentations. I dedicate my prizes, Excellence in Football Research Award (ECSS congress, Malmo, Sweden) and Young Investigator Award (ECSS congress, Vienna, Austria), to you.

My gratitude and a special thanks also go to my supervisor during many years of study, Prof. Jens Bangsbo, for concise and excellent guidance throughout the process, for your trust in me during experimental and analytical phases, and the opportunity to perform an exciting, invasive human experiment that I have wanted to do for many years.

I would also like to render my deepest gratitude to Prof. Robyn Murphy for invaluable inspiration, supervision, and collaboration, for teaching me the single-fibre technique, for many insightful discussions of research ideas over protein and mRNA data, and for welcoming me in your lab.

Finally, I would like to specifically render thanks to the following people for their excellent advice, assistance and inputs throughout the thesis:

Victoria University (Australia): Michael J. McKenna for excellent feedback on the thesis and paper III, and for sincere interest in my research. Christos G. Stathis for our analyses of metabolites for paper I and great discussions and company. James R. Broatch for good advice and being a great teammate on the 5-a-side soccer team, Jujiao Kuang for introducing me to RT-PCR and the lab, Weiliang Chung, Xu Yan, Cian McGinley, Jackson Fyfe, Cesare Granata, Rodrigo da Silva Fermino de Oliveira, Amanda Genders, Victoria Wyckelsma, and Nick Jamnick.

University of Copenhagen (Denmark): Thomas G. P. Gunnarsson, Morten Hostrup, Michael Nyberg, Kasper Eibye, Mads S. Lindskrog, Casper Skovgaard, Martin Thomassen, and Jens Jung Nielsen.

La Trobe University (Australia): Barneby Patrick Frankish, Stefan Georg Wette, Hongyang Xu, Rene Yen, and Heidy Latchman.

And the students on my projects for their great efforts during experiments, participant training, and/or laboratory analyses: Erch Selemi, Villads Rasmussen, Hans M. Voldbye, Peter Munck Sørensen, Tobias Buk Jørgensen, Daniel Lindsten, Eli Nolsøe Leifsson, Jesper Kirkebjerg, and Casper Bjerre Olsen.

The work was funded by an international postgraduate research scholarship (granted by Victoria University), the Danish Ministry of Culture, and Team Danmark (Danish Elite Sport Organisation).

Preface

Many important scientific contributions to the topic of potassium ion (K^+) handling by skeletal muscle have emerged since the discovery in 1938 that the loss of K^+ from excited myocytes is related to elevated electrical activity (Fenn, 1938). Many experiments using animal models, and a substantial number of human studies over the years, have consistently shown that the ability to maintain K^+ homeostasis in skeletal muscle is essential for physical function. This is underpinned by observations in humans that the muscle capacity for K^+ handling is reduced in various disease states, such as diabetes and heart failure (Kjeldsen *et al.*, 1987; Nishida *et al.*, 1992; Clausen, 1998; Harmer *et al.*, 2006; Galuska *et al.*, 2009), but can be improved by exercise training (Nielsen *et al.*, 2004; Gunnarsson *et al.*, 2013). Despite this evidence, the cellular mechanisms that drive these adaptations in humans are still poorly understood.

A main focus of the present thesis was to identify factors that play a significant role in acute and long-term regulation of muscle K^+ handling in humans. In the process of identifying these factors, this thesis has exploited alternative training approaches, such as blood flow restriction, altitude exposure, and cold-water immersion, to manipulate levels of metabolic, ionic and oxidative stress; these are all stimuli that have been shown to regulate the function and abundance of K^+ transport systems *in vitro*.

A secondary focus of this thesis was to identify limitations in currently used methods for analysing proteins in different fibre types of human skeletal muscle biopsies. This led to the development of a novel method for identifying the fibre type of individual fibre segments from human muscle biopsies, which is presented in paper IV. It is my hope that this new method will pave the way for more laboratories to undertake fibre-type-specific analyses due to the reduced costs and labour time associated with experiments using this new method.

The thesis is based on experimental work conducted in the period 2014 to 2017, with the collaboration of the following three institutions:

- Institute for Health and Sport, Victoria University, Melbourne, Australia.
- Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark.
- Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Australia.

March 2018
Danny Christiansen

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List of manuscripts

The current thesis is based on the following papers. The papers will be referred to in the thesis by their roman numerals. Abstracts from congress will also be highlighted where appropriate.

I: **Christiansen D**, Murphy RM, Bangsbo J, Stathis CG, Bishop DJ (2018): Increased *FXYD1* and *PGC-1α* mRNA after blood flow-restricted running is related to fibre type-specific AMPK signalling and oxidative stress in human muscle. *Acta Physiol* (Oxf). 2018 Jan 31. doi: 10.1111/apha.13045. Impact factor = 5.93.

II: **Christiansen D**, Eibye KH, Rasmussen V, Voldbye HM, Hostrup M, Gunnarsson TGP, Thomassen M, Lindskrog MS, Skovgaard C, Nyberg M, Bishop DJ, Bangsbo J (2018): Cycling with blood flow restriction improves performance and muscle K+ regulation and alters the effect of antioxidant infusion in humans. *J Physiol. 2019* Mar 7. doi: 10.1113/JP277657. [Epub ahead of print]. Impact factor = 5.037.

III: **Christiansen D**, David J. Bishop, James R. Broatch, Jens Bangsbo, Michael J. McKenna, Robyn M. Murphy (2018): Cold-water immersion after training sessions: effects on fiber type-specific adaptations in muscle K+ transport proteins to sprint-interval training in men. *J Appl Physiol* (1985). 2018 Aug 1;125(2):429-444. doi: 10.1152/japplphysiol.00259.2018. Impact factor = 3.256

IV: **Christiansen D**, MacInnis MJ, Zacharewicz E, Xu H, Frankish BP, Murphy RM (2019): A fast, reliable and sample-sparing method to identify fibre types of single muscle fibres. Accepted, Scientific Reports, March 8. Impact factor = 4.609 (5-year).

V: **Christiansen D** (2018): Molecular stressors underlying exercise training-induced improvements in K+ regulation during exercise and Na+ ,K+ -ATPase adaptation in human skeletal muscle. Acta Physiol (Oxf). 2019 Mar;225(3):e13196. doi: 10.1111/apha.13196. Impact factor = 5.93.

Awards and grants

Excellence in Football Research Award. Oral presentation, 2nd prize. *European College of Sport Science (ECSS) congress*, Sponsored by Aspetar. May 2015. Malmö, Sweden.

Young Investigator Award. Oral presentation, 4th prize. *European College of Sport Science (ECSS) congress*, July, 2016. Vienna, Austria.

Research grant. The effect of ischaemic exercise training on performance during repeated intense exercise and adaptations in the muscle ion transport system. FPK.2015-0017. *Danish Ministry of Culture*. July 2015. Copenhagen, Denmark.

List of published abstracts

I: **Christiansen D**, Broatch JR, Bishop DJ (2015): The effect of cold-water immersion on the change in skeletal muscle Na^+,K^+-ATPase genes following intense intermittent exercise in humans. *European College of Sport Science (ECSS) congress*. May. Malmö, Sweden.

II: **Christiansen D**, Murphy RM, Broatch JR, Bangsbo J, McKenna MJ, Bishop DJ (2015): Fibre-type specific changes to skeletal muscle Na^+,K^+-ATPase and FXYD1 following postexercise cold-water immersion in humans. *Australian Physiol Society (AuPS) conference*. November. Hobart, Tasmania.

III: **Christiansen D**, Murphy RM, Bishop DJ (2016): Fibre type-dependent activation of human muscle Ca^{2+}, ATP and ROS-sensitive signalling pathways by exercise is modified by ischaemia: Role in fibre type-specific regulation of mitochondrial and ion transport genes. *European College of Sport Science (ECSS) congress*. July. Vienna, Austria.

IV: **Christiansen D**, Murphy RM, Bishop DJ (2017): Ischaemic exercise enhances mitochondrial and ion transport gene adaptations in trained human skeletal muscle: Role of cellular redox state, AMPK and CaMKII signalling. *Tairyoku kagaku. Japanese journal of physical fitness and sports medicine* 66(1):75-75. January. DOI: 10.7600/jspfsm.66.75. Morioka, Japan. Invited presentation.

V: **Christiansen D** (2017): How can we improve the training response and intense exercise performance using BFR training? *Inspire Convention*. August. Nyborg, Denmark. Invited presentation.

VI: **Christiansen D**, Murphy RM, Bangsbo J, Bishop DJ (2017): Ischaemia promotes the exercise-stimulated muscle gene response associated with mitochondrial protein content and glucose transport in healthy men. *Cell*. Symposium on Exercise Metabolism. May. Gothenburg, Sweden.

VII: **Christiansen D** & Bansgbo J (2018): Blood-flow-restricted training augments improvements in muscle K^+ handling, antioxidant capacity and exercise performance in men. *American College of Sports Medicine (ACSM)*. May. Minneapolis, Minnesota, USA.

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CHAPTER I: Introduction

In this chapter, the scientific rationale for the experiments described in the current thesis is examined. An updated and improved version of this chapter has been published (Paper V).

1.1. Regulation of K^+ homeostasis and Na^+,K^+-ATPase function, content, and isoform abundance in human skeletal muscle

At the onset of muscle contraction, rapid and marked perturbations in muscle intracellular and interstitial concentrations of ions (K^+, Na^+, Ca^{2+}, Cl^-, H^+, lac^-) occur, which can both facilitate and constrain muscle activity (Sahlin, 1986; Lindinger & Sjogaard, 1991; Westerblad *et al.*, 1991; Bangsbo *et al.*, 1996; Nielsen *et al.*, 2001; de Paoli *et al.*, 2007). Strenuous physical exertion raises muscle interstitial K^+ concentration ($[K^+]_{int}$) by up to 2.5 to 3 fold in humans, which has been linked with impaired muscle force development (Bangsbo *et al.*, 1996; Juel *et al.*, 2000; Nordsborg *et al.*, 2003b; Nielsen *et al.*, 2004; Gunnarsson *et al.*, 2013). This may be caused in part by depolarisation of the cell membrane (Sjogaard *et al.*, 1985; Lindinger & Heigenhauser, 1991; McKenna *et al.*, 2008), but may also be due to increased activation of group III/IV afferent nerve fibres by K^+ in the interstitial space, resulting in diminished central motor drive to contracting muscle fibres (Hnik *et al.*, 1969; Amann *et al.*, 2013). Furthermore, K^+ efflux from exercising muscles to the bloodstream may impair myocardial excitation (Kjeldsen, 1991). On the other hand, K^+ acts as an arteriolar vasodilator (Kjellmer, 1965), which may aid blood perfusion of muscle fibres under certain conditions (Hellsten *et al.*, 2012). Increased $[K^+]_{int}$ has also been shown to activate the exercise pressor reflex by stimulating afferent nerve fibres, resulting in an elevated heart rate and rate of ventilation (Lindinger & Sjogaard, 1991; Paterson, 1992). These actions can both aid delivery of substrate for metabolism and removal of metabolic by-products from exercising or recovering muscle fibres. Thus, maintenance of K^+ concentrations in the musculature plays an integrative role in regulating muscle contractile function and thus exercise tolerance.

Intense exercise elicits marked increases (up to ~12 $mmol \cdot L^{-1}$) in muscle $[K^+]_{int}$ (Nordsborg *et al.*, 2003b; Mohr *et al.*, 2004) and intramyocellular Na^+ concentration (Overgaard *et al.*, 1997), both of which have been linked to muscle fatigue (Clausen, 2003). These ionic perturbations are counteracted primarily by increasing the activity of the Na^+,K^+-ATPase, which actively transports three Na^+ out and two K^+ into the muscle fibres for each ATP molecule hydrolysed (Lavoie *et al.*, 1997; Overgaard *et al.*, 1997). Thus, the Na^+,K^+-ATPase is critical for maintenance of muscle Na^+ and K^+ homeostasis, excitability, and contractile function (McKenna *et al.*, 2008). The Na^+,K^+-ATPase consists of a catalytic α subunit, a structural and regulatory β subunit, and an accessory γ subunit, named phospholemman (FXYD), which is coexpressed with the α subunit and is required

for basal Na^+,K^+-ATPase function (Reis *et al.*, 2005; Bibert *et al.*, 2008; Manoharan *et al.*, 2015). In human skeletal muscle, each of the α and β subunits exists as three different isoforms (α_{1-3} and β_{1-3}) (Wyckelsma *et al.*, 2015), whereas FXYD1 is the only isoform of the γ subunit expressed in this tissue (Floyd *et al.*, 2010). The total capacity for Na^+ and K^+ transport by the Na^+,K^+-ATPase is in part determined by the number of active αβ-heterodimer complexes at the cell surface (Rasmussen *et al.*, 2008). But the relative recruitment or activation of different α isoforms may also be influential, because of the distinct ion transport properties of these isoforms (Radzyukevich *et al.*, 2004; Radzyukevich *et al.*, 2013). In addition, FXYD1 also participates in the complex regulation of Na^+,K^+-ATPase function by protecting this system against oxidative damage (Bibert *et al.*, 2011) and by modulating Na^+,K^+-ATPase Na^+ affinity (Reis *et al.*, 2005; Ingwersen *et al.*, 2011). Thus, each of these subunits appears to be functionally relevant and their recruitment important for the net transport of K^+ and Na^+ across the plasma and T-tubular membranes.

From the cross-sectional data summarised in Table 1.1 and Fig. 1.1 (next page), it is clear that exercise training, regardless of whether it is performed at/below (primarily aerobic) or above (highly anaerobic) the intensity eliciting VO_{2max}, is an effective stimulus to increase both Na^+,K^+-ATPase content (11–15%; as determined by [^{3}H]-ouabain binding site content) and isoform abundance (5–44%; as quantified using western blotting) in humans. Aerobic training has also been shown to increase Na^+,K^+-ATPase maximal *in vitro* activity (4%; as assessed by the 3-O-MFPase method), whereas the effects of anaerobic training on this variable remains to be examined. The functional relevance of increases in Na^+,K^+-ATPase content, isoform abundance, and/or activity is evidenced by findings of concomitant reductions (5–8 %) in K^+ concentration in the bloodstream or in the muscle interstitial space during exercise, and simultaneous improvements (14–16%) in physical performance in many studies (Table 1.1). These adaptations may occur rapidly, with decreases in venous blood [K^+] (5%) and increases in Na^+,K^+-ATPase activity (41%), content (9–14 %), and isoform abundance (27–113%, α_1, α_2 and β_1), reported after only six to ten days of training (Green *et al.*, 1993; Green *et al.*, 2004; Benziane *et al.*, 2011). However, changes in functional variables, such as muscle K^+ release, venous [K^+], and Na^+,K^+-ATPase activity, with both one type or different types of training appear to be dissociated from those of Na^+,K^+-ATPase content or isoform abundance in many studies (Nielsen *et al.*, 2004; Aughey *et al.*, 2007; Mohr *et al.*, 2007; Green *et al.*, 2008; Skovgaard *et al.*, 2017a). For example, three consecutive days of continuous training (2 hour cycling at 60% VO_{2max}) increased (12%) [^{3}H]-ouabain binding site content, but decreased (34%) 3-O-MFPase activity in recreationally-active subjects (Green *et al.*, 2008). In similarly trained men, muscle abundance of Na^+,K^+-ATPase α_1 (29%) and α_2 (15%) increased and $[K^+]_{int}$ decreased (-27%), whereas K^+ release from exercising muscles remained unaltered after seven weeks of intense interval training (15 x 1 min at 150% of leg VO_{2max}) (Nielsen *et al.*, 2004). Further, six to eleven weeks

of anaerobic training resulted in an increased β_1 abundance (39 to 58%), despite a higher (14%) venous blood [K^+] during exercise in highly-trained runners (Skovgaard *et al.*, 2017a).

Thus, amongst the published human studies to date, there is a consensus that both aerobic and anaerobic training are powerful stimuli for enhancing K^+ regulation and Na^+,K^+-ATPase function, content, and isoform abundance, although increases in isoform abundance were highly variable among studies. Furthermore, increases in these variables with training are often temporally associated with improvements in one or more aspects of physical performance. However, in many studies, training-induced alterations in plasma [K^+] or Na^+,K^+-ATPase activity were dissociated from changes in Na^+,K^+-ATPase protein expression. This paradox remains poorly addressed in the literature. In addition, based on the cross-sectional data, it remains unclear what is the best training strategy to maximise adaptations in skeletal muscle K^+ regulation, and Na^+,K^+-ATPase content and function, in humans.

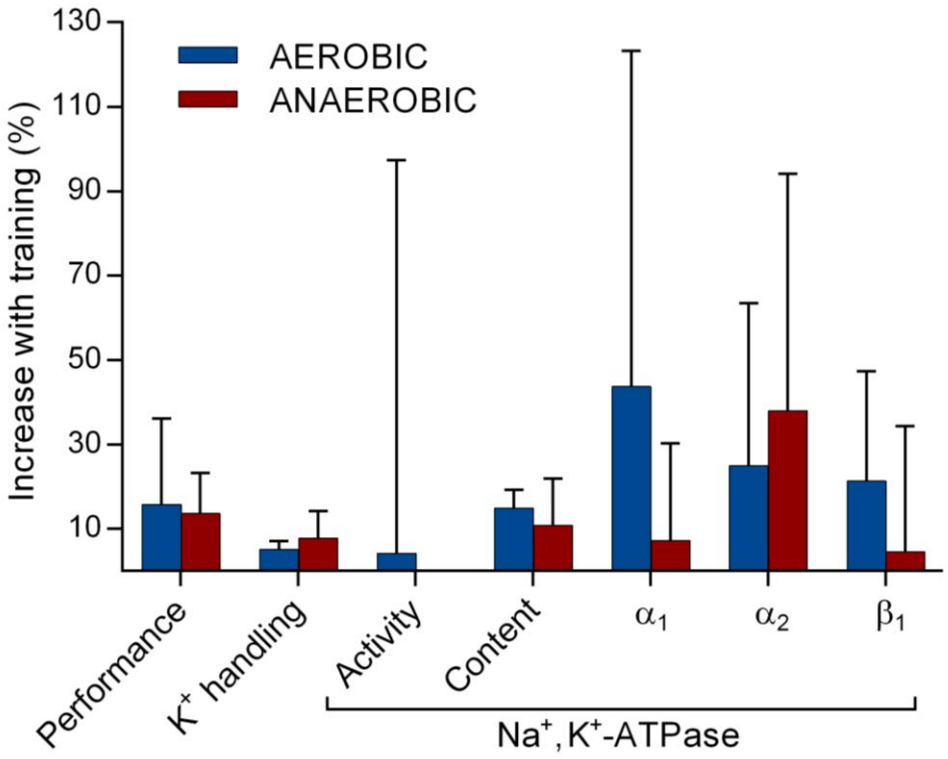

Figure 1.1. Effects of different types of training on intense exercise performance, K^+ handling, and skeletal muscle Na^+,K^+-ATPase function, content, and isoform (α_1, α_2, β_1) abundance in humans. Aerobic = Training at intensities ≤ VO_{2max} (*n* = 13 interventions) (Green *et al.*, 1993; Madsen *et al.*, 1994; Evertsen *et al.*, 1997; Green *et al.*, 1999a; Green *et al.*, 1999b; Green *et al.*, 2004; Aughey *et al.*, 2007; Green *et al.*, 2008; Benziane *et al.*, 2011; Edge *et al.*, 2013), Anaerobic = Training at intensities > VO_{2max} (*n* = 8 interventions) (McKenna *et al.*, 1993; Nielsen *et al.*, 2004; Harmer *et al.*, 2006; Mohr *et al.*, 2007; Iaia *et al.*, 2008; Bangsbo *et al.*, 2009;

Gunnarsson *et al.*, 2012). Note that measurement of Na^+,K^+-ATPase activity was not included in studies on anaerobic training. Data are expressed as means + 95% confidence intervals.

1.2. Fibre-type-specific expression of Na^+,K^+-ATPase isoforms in skeletal muscle and adaptations to exercise training

Human skeletal muscle is a heterogeneous tissue consisting of fibres with distinct metabolic and ionic properties. Due to these differences, fibres can be defined according to their content of proteins with different functions. For example, fibres may be characterised by their content of myosin heavy chain (MHC) isoforms as type I, IIa, IIx, or hybrid if containing multiple MHC isoforms (e.g. I/IIa or IIa/IIx). In comparison to type II (fast-twitch) fibres, type I (slow-twitch) fibres have a slower rate of force development and sarcoplasmic reticulum (SR) Ca^{2+} release and uptake kinetics, altered glycogen utilisation, possess more mitochondria, and are more fatigue resistant (Essen *et al.*, 1975; Karlsson *et al.*, 1981; Ball-Burnett *et al.*, 1991; Greenhaff *et al.*, 1993; Lamboley *et al.*, 2013, 2014). In animals, differences in the capacity for Na^+/K^+ handling among different skeletal muscles have also been observed, and this has been associated with a different expression of Na^+,K^+-ATPase isoforms between different fibre types (Juel, 2009; Kristensen & Juel, 2010b, a). In humans, α_2 abundance was found to be higher in type II compared to type I skeletal muscle fibres in recreationally-active men (Thomassen *et al.*, 2013). Furthermore, FXYD1 was more highly expressed in type I compared to type II muscle fibres in sedentary rats (Reis *et al.*, 2005; Juel, 2009), and in humans its phosphorylation state was rapidly increased in type II but not in type I muscle fibres after a single session of intense exercise (Thomassen *et al.*, 2013). Collectively, these studies suggest that expression of Na^+,K^+-ATPase isoforms and FXYD1 activation (by phosphorylation) may be altered by exercise in a fibre-type-dependent manner, which may significantly impact K^+ regulation in the musculature. Nevertheless, in most human training studies, Na^+,K^+-ATPase-isoform abundance was quantified in fibre-type heterogeneous (whole-muscle) samples (Table 1.1), indicating that important changes in isoform levels could have been overlooked. Another concern is that protein abundance was quantified using fractionated samples in many previous studies (Green *et al.*, 2004; Nielsen *et al.*, 2004; Iaia *et al.*, 2008; Thomassen *et al.*, 2010; Gunnarsson *et al.*, 2012). This is an issue, because a proportion of the protein being analysed may be inadvertently lost by fractionation (Murphy & Lamb, 2013). In addition, some of the studies did not take into consideration blot linearity, making it impossible to know if protein bands were saturated and thus should be excluded from analysis (Mollica *et al.*, 2009). Moreover, few studies validated their antibodies, for example by loading positive and/or negative control tissues. Limitations in methodology for protein quantification could thus be one explanation for the dissociation between changes in expression of Na^+,K^+-ATPase isoforms and those of the capacity for K^+ regulation observed in the literature (Table 1.1). On this basis, assessment of effects of different types of training on the abundance of Na^+,K^+-

ATPase isoforms in human skeletal muscle with the use of improved methodology (i.e. no fractionation, antibody validation, fibre type-specific analysis, and normalisation to a standard curve) appears heavily warranted.

At the time of commencing the present thesis, only one published study had examined the effects of training on Na^+,K^+-ATPase-isoform abundance in different muscle fibre types in humans. In this study, four weeks of sprint-interval training increased the abundance of the β_1 isoform in type II fibres only (Wyckelsma *et al.*, 2015), whereas the catalytic isoforms remained unchanged. However, this study applied a training volume ~12-18 times lower than that of other human studies that reported increases in α-isoform abundance in whole-muscle samples (Mohr *et al.*, 2007; Iaia *et al.*, 2008; Bangsbo *et al.*, 2009). It is thus possible that the training period was too short in the study by Wyckelsma *et al.* (2015) to invoke changes in α-isoform levels in any fibre type. Within the past two years, two other human studies investigating changes in Na^+,K^+-ATPase-isoform abundance at the fibre-type level were published. In one of these studies, four weeks of resistance training (after preceding period of disuse) elevated the content of α_2 (76%) and α_3 (143%) in type I fibres, and α_1 (79%) and β_1 (35%) in type II fibres, from the skeletal muscle of sedentary subjects. However, isoform abundance was not altered in whole-muscle samples from the same biopsies (Perry *et al.*, 2016). In the other recent study, α_2 abundance only increased (30%) in type II fibres after twelve weeks of high-intensity aerobic training (4 x 4-min cycling bouts at 90 to 95% HR_{peak}, separated by 4 min at 50 to 60% HR_{peak}), which was associated with elevated (11%) [^{3}H]-ouabain binding site content. But again, whole-muscle abundance of Na^+,K^+-ATPase isoforms remained unchanged with the training intervention (Wyckelsma *et al.*, 2017). Collectively, these findings stress the need for fibre-type specific protein analysis. In the three human studies published to date that have examined effects of training on Na^+,K^+-ATPase expression in type I and II muscle fibres, the capacity for K^+ regulation in the trained musculature was not assessed, making implications of their fibre-type specific protein results for muscle K^+ handling challenging (or impossible) to delineate. Further research is clearly warranted to clarify the relationship between training-induced adaptations in fibre-type-specific protein abundance and the capacity for K^+ handling in human skeletal muscle.

Fibre-type-specific protein analyses of human muscle tissue go back several decades from the early classification of fibre types according to myofibrillar ATPase activity in the 70s (Brooke & Kaiser, 1970; Guth & Samaha, 1970), and the recognition of myosin heavy chain isoforms for determination of fibre types in the 80s and early 90s (Biral *et al.*, 1988; Staron, 1991; Larsson & Moss, 1993; Ennion *et al.*, 1995), to recent developments of fibre-type-identification techniques predicated on western blotting (Murphy, 2011a). These methods paved the way for novel discoveries at the fibre-type level. However, they are limited by tedious, costly, and time-consuming sample collection and analysis

steps, making fibre-type-specific studies of human muscle tissue a challenging task. Thus, it would be useful to develop a new method for easy and rapid fibre type identification of muscle fibres for use in both human and animal research.

Table 1.1 Effects of different types of training on performance, K^+ regulation, and skeletal muscle Na^+,K^+-ATPase content and function in humans

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	[^{3}H]ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
Aughey *et al.* (2007)	Male cyclists or triathletes (n=12) 65 mL/kg/min	2–3 sessions/wk for 3 weeks 8 x 5-min cycling at 80 % peak power output (1 min cycling at 100 W)	Peak power during incremental cycling to exhaustion ↑3%		3-O-MFPase activity ↑5%	0%	α_1, α_2, α_3: 0% β_1, β_2, β_3: 0%
Edge *et al.* (2013)	Recreationally-active women (n=6) 46 mL/kg/min	3 sessions/wk for 5 weeks 6-10 x 2 min at 140-190 % LT (1 min rest)	Power at VO_{2peak} ↑12%			↑22%	
Edge *et al.* (2013)	Recreationally-active women (n=6) 46 mL/kg/min	3 sessions/wk for 5 weeks 6-10 x 2 min at 140-190 % LT (3 min rest)	Power at VO_{2peak} ↑9%			↑26%	
Green *et al.* (1993)	Untrained men (n=9) 48 mL/kg/min	6 sessions over 6 days 2-h cycling at 60-65 % VO2max	Incremental cycling test to exhaustion (VO2max) ↑7%	Venous plasma $[K^+]$ ↓5%		↑14%	
Green *et al.* (1999a)	Healthy and untrained subjects (n=7), sex n/a 44.4 mL/kg/min	5–6 sessions/wk for 11 weeks 2-h cycling at 68 % VO2max	Incremental cycling test to exhaustion (VO2max) ↑15%			↑22%	
Green *et al.* (2004)	Untrained men (n=7) 46 mL/kg/min	6 sessions over 6 days 2-h cycling at 60-65 % VO2max			3-O-MFPase activity ↑41%	↑9%	α_1: ↑16% α_2: ↑9% β_1: ↑39%
Green *et al.* (2008)	Recreationally-active men (n=6) and women (n=6) 45 mL/kg/min	3 sessions over 3 days 2 h cycling at 60 % VO2max			3-O-MFPase activity ↑34%	↑12%	α_1: ↑46% α_2: ↑42% α_3: ↑31% β_1: ↑19%, β_2: ↑28% β_3: : ↑20%
Madsen *et al.* (1994)	Male runners (n=42) 55 mL/kg/min	3 sessions/wk for 6 weeks 25 min running at 93% HRmax	Running at pre-train 86 % VO2max to exhaustion (TTE) ↑75%			↑15%	
Benziane *et al.* (2011)	Healthy men (n=8) 61 mL/kg/min	4 sessions over 10 days 45-90 min cycling at 75 % VO2max and 6 x 5 min cycling at 90-100 % VO2max with 2 min cycling <40 % VO2max between bouts on alternate days		Venous plasma [K+] ↓5%			α_1: ↑113% α_2: ↑49% α_3: 0% β_1: ↑27%, β_2: 0%
Evertsen *et al.* (1997)	Male (n=11) and female (n=9) cross country skiers 73 and 58 mL/kg/min	7 sessions/wk for 20 weeks Running, roller- or cross-country skiing at moderate (40 min to 3 h at 60-70% VO2max) (86%) and high intensity (40 s to 7 min at 80-90% 2max for 10 min to 2 h) (14%)	20-min running time trial ↑2%			↑16%	

Table 1.1 Continued from previous page.

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	[^{3}H]ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
Evertsen *et al.* (1997)	Male (n=11) and female (n=9) cross country skiers 73 and 58 mL/kg/min	7 sessions/wk for 20 weeks Running, roller- or cross-country skiing at 80-90 % VO2max (10 min to 2 h) or 40 s to 7 min at similar intensity (83%) or at 60-70 % VO2max (17%)	20-min running time trial ↑4%			↑16%	
Green *et al.* (1999b)	Healthy men (n=9) VO_{2max} n/a	3 sessions/wk for 8 weeks 30-min one-legged cycling at 75-87% peak power output (first 6 weeks) and 5 x 3-min cycling at 100% pre-train peak power output (final 2 weeks)	Unilateral cycling to fatigue at 95 % VO2max ↑400%			↑14%	
Green *et al.* (1999b)	Healthy men (n=9) VO_{2max} n/a	3 sessions/wk for 8 weeks 30-min one-legged cycling at 75-87% peak power output (first 6 weeks) and 5 x 3-min cycling at 100% pre-train peak power output (final 2 weeks) in normobaric systemic hypoxia	Unilateral cycling to fatigue at 95 % VO2max ↑510%			↑14%	
Bangsbo *et al.* (2009)	Endurance-trained men (n=17) 63 mL/kg/min	2–3 sessions/wk for 6–9 weeks Addition of sessions of 8-12 x 30-s at 95% maximal speed (3 min rest)	Exhaustive supramaximal treadmill run at 130% VO2peak ↑36%	Venous plasma [K+] ↓6%			α_1: 0%, α_2: ↑68% β_1: 0%
Gunnarsson *et al.* (2012)	Danish 2nd Division male soccer players (n=16) 61 mL/kg/min	1 additional session/wk for 5 weeks Addition of a session of 6-9 x 30-s runs at 90-95 % maximal intensity (3 min)	incremental running time to exhaustion ↑11%				α_1: 0%, α_2: 0% β_1: ↓13%
Harmer *et al.* (2006)	type-1 diabetic men (n=5) and women (n=3) 3300 mL/min	3 sessions/wk for 7 weeks 4-10 x 30-s 'all-out' cycling sprints, 3-4 min rest	Incremental cycling test to exhaustion (VO2max and peak power) ↑11%	Venous plasma [K+] ↓7%		↑8%	
Harmer *et al.* (2006)	Healthy men (n=4) and women (n=3) 3170 mL/min	3 sessions/wk for 7 weeks 4-10 x 30-s 'all-out' cycling sprints, 3-4 min rest	Incremental cycling test to exhaustion (VO2max and peak power) ↑11%	Venous plasma [K+] ↓7%		↑8%	
Iaia *et al.* (2008)	Male runners (n=8) 56 mL/kg/min	3–4 sessions/wk for 4 weeks 8-12 x 30-s running bouts at 90-95 % of the max. speed attained during a 30-s 'all-out' run (3 min rest bt sprints)	running to fatigue at 130 % VO2max and incremental test to exhaustion ↑5%	Venous plasma [K+] ↓8%			α_1: ↑29% α_2: ↑16% β_1: 0%

Table 1.1 Continued from previous page.

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	[^{3}H]ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
McKenna *et al.* (1993)	Healthy, untrained men (n=6) 51 mL/kg/min	3 sessions/wk for 7 weeks 4-10 x 30-s maximal-intensity cycling sprints (2.5-4 min rest)	4 maximal 30-s sprints ↑6%	Venous plasma [K+] ↓19%		↑16%	
Mohr *et al.* (2007)	Healthy men (n=7) 49 mL/kg/min	3–6 sessions/wk for 8 weeks 8 x 30-s runs at 130 % VO2max, 1.5 min rest	incremental exercise to exhaustion ↑15%	Venous plasma [K+] ↓0%			α_1: 0% α_2: ↑68% β_1: ↑31%
Dela *et al.* (2004)	Healthy subjects (n=7) VO_{2max} n/a	3 sessions/wk for 6 weeks 3-4 sets of 10-12 reps at 50-80% 1RM, 1.5-2 min rest	Maximal leg press and knee-extensor force ↑77% and ↑29%				α_1: ↑37% α_2: ↑22% β_1: ↑33%
Dela *et al.* (2004)	Type-II diabetes patients (n=10) VO_{2max} n/a	3 sessions/wk for 6 weeks 3-4 sets of 10-12 reps at 50-80% 1RM, 1.5-2 min rest	Maximal leg press and knee-extensor force ↑75% and ↑42%				α_1: ↑45% α_2: ↑41% β_1: ↑47%
Green *et al.* (1999a)	Healthy and untrained subjects (n=9), sex n/a 45 mL/kg/min	3 sessions/wk for 12 weeks Resistance training: 3 x 6-8 1RM of squats, leg press, leg extensions	Incremental cycling test to exhaustion (VO2max) ↑0%			↑16%	
Medbo *et al.* (2001)	Male athletes (n=23) VO_{2max} n/a	1–3 sessions/wk for 12 weeks 5 x 4 squats at 50% 1RM (concentric phase) and 110-135 % 1RM (eccentric phase)	1RM squat and Number of squats at 70 % 1RM to exhaustion ↑7% and ↑29%			↑15%	
Perry *et al.* (2016)	Sedentary, healthy men (n=4) and women (n=2) 46 mL/kg/min	3 sessions/wk for 4 weeks 3-4 sets of 8-12 reps of leg-press, knee-extension, hamstring curls, and calf raises at 65-70 % 1RM (1 min rest)	Time to fatigue cycling at 85 % leg VO2 peak ↑31%	Venous plasma [K+] and Δ[K+]/work ratio ↓0%		0%	α_1, α_2, α_3: 0% β_1, β_2, β_3: 0%
Skovgaard *et al.* (2014b)	Endurance-trained male runners (n=12) 59 mL/kg/min	4 sessions/wk for 8 weeks Two sessions of 4-12 x 30-s 'all-out' (3 min rest) and two sessions of resistance training (3 sets of 8 reps at 15RM to 4 sets of 4 reps at 4 RM, squats, deadlift, leg press)	10-km run, 1500 m run, Yo-Yo IR2, VO2max ↑4%, ↑6%, ↑44% and ↑0%				α_1, α_2: 0% β_1: 0%
Skovgaard *et al.* (2017a)	Endurance-trained male and female runners (n=11) 59 and 50 mL/kg/min	6 sessions/wk for 6 wk Maintained high-frequency SET (four sessions of 8-12 x 30-s 'all-out' runs, 3.5 min rest) and moderate-intensity running (two sessions of 30-60 min running at 60-80 % HRmax)	TTE during intense exercise ↑12%	Venous plasma [K+] ↓0%			α_1, α_2: 0% β_1: ↑39%

Table 1.1 Continued from previous page.

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	$[^3H]$ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
Skovgaard *et al.* (2017a)	Endurance-trained male and female runners (n=7) 59 and 50 mL/kg/min	4 sessions/wk for 12 weeks Maintained low-frequency SET (two sessions of 10 x 30-s 'all-out' runs, 3.5 min rest) and moderate-intensity running (two sessions of 30-60 min running at 60-80 % HRmax)	TTE during intense exercise ↑16%	Venous plasma [K+] ↓14%			α_1, α_2: 0% β_1: ↑58%
Skovgaard *et al.* (2017b)	Endurance-trained male runners (n=8) 59 mL/kg/min	4 sessions/wk for 6 weeks Two sessions of 4-12 x 30-s 'all-out' (3 min rest) and two sessions of running for 30-60 min at 60-80 % HRmax	Incremental running to exhaustion (VO2max) ↑20%				α_1, α_2: 0% β_1: 0%
Thomassen *et al.* (2010)	Elite soccer players (n=18) 55 mL/kg/min	4.5 sessions/wk for 2 weeks Small-sided games (8 x 2 min at 88 % HRmax, 1 min rest) and 10-12 x 25-30-s 'all-out' runs and 16 x 40-60-s runs at 84 % HRmax, 40-60-s rest	RSA, total sprint time, fastest sprint time, Yo-Yo IR2 ↑3%, ↑2%, ↑0%, and ↑0%				α_1: 0%, α_2: ↑15% β_1: 0%
Nielsen *et al.* (2004)	Healthy men (n=6) 50 mL/kg/min	3–5 sessions/wk for 7 weeks 15 x 1 min at 150% thigh VO2peak (3 min rest)	Incremental exercise to exhaustion ↑28%	Muscle interstitial [K+] ↓27%			α_1: ↑29%, α_2: ↑15% β_1: 0%
Nielsen *et al.* (2004)	Healthy men (n=6) 50 mL/kg/min	3–5 sessions/wk for 7 weeks 15 x 1 min at 150% thigh VO2peak (3 min rest)	Incremental exercise to exhaustion ↑28%	Leg K+ release ↓0%			α_1: ↑29%, α_2: ↑15% β_1: 0%
Mohr *et al.* (2007)	Healthy men (n=6) 50 mL/kg/min	3–6 sessions/wk for 8 weeks 15 x 1 min at 150% thigh VO2peak (3 min rest)	Incremental exercise to exhaustion ↑28%	Venous plasma [K+] ↓0%			α_1: 0%, α_2: 0% β_1: ↑34%

Thirty human training studies included measurement of two or more of the following factors: Na^+,K^+-ATPase activity, content, whole-muscle isoform abundance, and K^+ regulation. Two studies were excluded as their main focus was on the effect of the environment (altitude exposure) rather than training (Aughey *et al.*, 2006; Nordsborg *et al.*, 2012), and one because training sessions were performed on a single day (Green *et al.*, 2007). Searches were conducted on Pubmed, Science Direct, Google Scholar, and Web of Science using words such as: Na^+,K^+-ATPase, performance, training, human muscle. Manual searches were also performed using reference lists of published studies.

1.3. Regulation of Na^+,K^+-ATPase-isoform mRNA in human skeletal muscle by exercise

Changes in steady-state protein abundance may often be partly determined by variance in mRNA levels (Li *et al.*, 2014). A single exercise session increases the mRNA of a growing number of genes (Mahoney *et al.*, 2005; Little *et al.*, 2011; Vissing & Schjerling, 2014), and these increases may often occur prior to upregulation of protein content in human skeletal muscle (Perry *et al.*, 2010). Thus, measurement of mRNA responses to a single exercise session can often provide valuable insights into the potential of a given training strategy to modulate abundance of proteins and ultimately their function. However, this assumption should be carefully regarded, because of the complex process that underpins upregulation of protein levels. Increases in protein are not exclusively a result of elevated mRNA availability, but also depends on efficient mRNA translation and protein stabilisation (synthesis relative to degradation). Further complicating interpretation, mRNA availability is rapidly altered according to mRNA transcription, alternative splicing, synthesis, decay, and translation (Garneau *et al.*, 2007).

The cross-sectional data summarised in Table 1.2 support that Na^+,K^+-ATPase isoforms (α_{1-3} and β_{1-3}) may be regulated at the mRNA level by a single session of exercise in humans. By comparing the responses in mRNA expression to a single exercise session (Table 1.2) with corresponding changes in protein content to a period of exercise training (Table 1.1), it could be argued that effects of an exercise session on Na^+,K^+-ATPase mRNA transcript levels reflect changes in corresponding isoform protein abundance after a period of the same type of training. For example, an increase (3 fold) in muscle α_1 mRNA was observed in recovery from a single session consisting of fifteen 1-min exercise bouts at 150% of leg VO_{2max} separated by 3 min of rest (Nordsborg *et al.*, 2003a), whereas the same training protocol performed 3 to 5 times per week for seven weeks resulted in an elevated (29%) α_1 protein abundance in what appears to be the same individuals (Nielsen *et al.*, 2004). Further, increases in α_1 (2 fold) (Murphy *et al.*, 2006b), α_2 (1.8 fold), and α_3 mRNA (3.3 fold) (Murphy *et al.*, 2008) have been reported after a session of continuous aerobic exercise (45 to 55 minutes at 71 to 75% of VO_{2max}). In comparison, 6 days to 11 weeks of regularly performing continuous aerobic training (2 hours at 60 to 65% VO_{2max}) resulted in elevated α_1 (16%) and α_2 (9%) protein abundance and [^{3}H]-ouabain binding site content (9 to 14%) (Green *et al.*, 1993; Green *et al.*, 1999a; Green *et al.*, 2004). Further, an increase in [^{3}H]-ouabain binding (14%) was reported after several weeks of intense aerobic interval training with one leg (5 x 3-min of unilateral cycling at 100% of pre-train aerobic peak power), whereas one session with the same type of exercise (5 x 2-5 min at 56 ± 5 W and 60 kick/min separated by 3 min of rest) elevated the mRNA levels

of α_1 (3.8 fold) and α_2 (2.4 fold) in recreationally-active subjects. However, an association between mRNA and protein adaptations is not a universal finding. For example, no change in α_2 mRNA was observed after a session of intense interval exercise (Nordsborg *et al.*, 2003a), despite an increase in α_2 protein (15%) after seven weeks of regularly performing the same type of exercise (Nielsen *et al.*, 2004). In addition, while the effects of sprint-interval training on protein abundance of Na^+,K^+-ATPase isoforms ($\alpha_{1\text{-}3}$ and $\beta_{1\text{-}3}$) are well-investigated, with reported increases of 8 to 29% in either α_1 or α_2 protein, or [^{3}H]-ouabain binding site content (McKenna *et al.*, 1993; Harmer *et al.*, 2006; Mohr *et al.*, 2007), no studies have examined the impact of this exercise modality on Na^+,K^+-ATPase-isoform mRNA content (Table 1.2).

In summary, there are some indications that the accumulative effects of repeated exercise-induced changes in mRNA is an important determinant of net protein turnover of Na^+,K^+-ATPase isoforms after several weeks of training. However, it is clear from current literature that interpretation of implications of variance in mRNA for the net protein turnover with training is constrained primarily by two factors: 1) use of different exercise protocols to examine mRNA and protein responses, and 2) mRNA and protein analyses not being performed in samples from the same subjects. In addition, as highlighted earlier in this chapter, limitations in methodology for protein analysis may have confounded some of the previous protein outcomes, making firm conclusions about an association between mRNA and protein responses Na^+,K^+-ATPase isoforms challenging. Moreover, it is evident from the literature summary that no study has examined the impact of exercise on FXYD1 mRNA content in human skeletal muscle.

Table 1.2 Literature summary of effects of a single exercise session on mRNA content of Na^+,K^+-ATPase isoforms in human skeletal muscle

Study	Subjects	Exercise protocol	Metabolic changes	Na^+,K^+-ATPase isoforms					
				α_1	α_2	α_3	β_1	β_2	β_3
Aughey *et al.* (2007) Metabolic changes from Stepto *et al.* (2001)	Well-trained cyclists and triathletes (n=12) *Pre training* 66 mL/kg/min	8 x 5 min at ~85% VO_{2peak} (80% PPO) separated by 1 min at 100 W (~1.3 W/kg)	Peak muscle lactate: ~32.7 mmol/L Minimum muscle pH: ~7.01 Peak blood lactate ~5-6 mmol/L Minimum blood pH: ~7.32 Peak blood K^+: ~5.4	0 h: ~3.0↑	0 h: ~3.4↑	0 h: ~5.0↑	0 h: →	0 h: →	0 h: →
Aughey *et al.* (2007) Metabolic changes estimated from Stepto *et al.* (2001)	Well-trained cyclists and triathletes (n=12) *Post training* 66 mL/kg/min	8 x 5 min at ~85% VO_{2peak} (80% PPO) separated by 1 min at 100 W (~1.3 W/kg)	Peak muscle lactate: ~32.7 mmol/L Minimum muscle pH: ~7.01 Peak blood lactate ~5-6 mmol/L Minimum blood pH: ~7.32 Peak blood K^+: ~5.4	0 h: ~4.0↑	0 h: →	0 h: ~4.0↑	0 h: →	0 h: ~2.7↑	0 h: →
Mahoney *et al.* (2005)	Healthy males (n=14) VO_{2max} n/a	~75 min at 60-85% VO_{2peak} (exhaustion)	n/a	n/a	n/a	n/a	n/a	n/a	3 h: ~2.7↑
Murphy *et al.* (2004) VO2 estimated from Radegran and Saltin (2000)	Healthy males (n=8) and females (n=7) 51 mL/kg/min	~5 min 52 s at ~51% leg VO_{2peak} (~40% of maximal work output) (exhaustion)	n/a	0 h: → 3 h: → 24 h: ~2.5↑	0 h: → 3 h: ~3.5↑ 24 h: →	0 h: ~2.4↑ 3 h: → 24 h: →	0 h: → 3 h: → 24 h: →	0 h: ~1.7↑ 3 h: → 24 h: →	0 h: → 3 h: → 24 h: →
Murphy *et al.* (2006b)	Healthy males (n=6) and females (n=5) 62 mL/kg/min	54.5 min at 75 % VO_{2peak} (exhaustion)	Plasma volume ↓ by ~11.2 %; Plasma $[K^+]$ ↑ from 4.25 (rest) to 5.3 mmol/L (exercise peak)	0 h: → 3 h: → 24 h: ~2.0↑	0 h: → 3 h: → 24 h: →	0 h: ~2.2↑ 3 h: → 24 h: →	0 h: → 3 h: → 24 h: →	0 h: ~1.9↑ 3 h: → 24 h: →	0 h: → 3 h: → 24 h: →
Murphy *et al.* (2008) Metabolic changes from Medved *et al.* (2004a)	Healthy males (n=8) 66 mL/kg/min	45 min at 71 % VO_{2peak}	Mixed-venous plasma K^+: 4.8 mmol/L H^+: ~45.2 mmol/L Na^+: ~143 mmol/L	0 h: →	0 h: ~1.8↑	0 h: ~3.3↑	0 h: ~2.4↑	0 h: ~1.6↑	0 h: →
Nordsborg *et al.* (2003a)	Healthy males (n=6) *Untrained leg* 50 mL/kg/min	15 x 1 min at 150 % leg VO_{2peak} separated by 3 min of rest	n/a	0 h: ~3.0↑ 1 h: ~3.0↑ 3 h: ~3.0↑ 5 h: → 24 h: →	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	n/a	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	n/a	n/a
Nordsborg *et al.* (2003a)	Healthy males (n=6) *Trained leg* 50 mL/kg/min	15 x 1 min at 150 % leg VO_{2peak} separated by 3 min of rest	n/a	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	n/a	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	n/a	n/a
Nordsborg *et al.* (2005)	Recreationally active males (n=8) VO_{2max} n/a	5 x 2-5 min at 56 ± 5 W and 60 kick/min separated by 3 min of rest	Blood lactate = 4.2 ± 2.2 mM Epinephrine: 0.3 ± 0.1 nM Norepinephrine: 0.8 ± 0.3 nM	0 h: → 1 h: ~3.8↑ 3 h: ~3.5↑ 5 h: ~2.5↑	0 h: → 1 h: ~2.1↑ 3 h: ~2.4↑ 5 h: ~1.9↑	n/a	0 h: → 1 h: ~2.8↑ 3 h: ~2.7↑ 5 h: ~2.2↑	0 h: → 1 h: → 3 h: → 5 h: →	0 h: ~1.9↑ 1 h: ~2.2↑ 3 h: ~3.1↑

5 h: ~2↑

Table 1.2 Continued from previous page.

Study	Subjects	Exercise protocol	Metabolic changes	Na+,K+-ATPase isoforms α_1	α_2	α_3	β_1	β_2	β_3
Nordsborg *et al.* (2005)	Recreationally active males (n=8) VO_{2max} n/a	5 x 2-5 min at 56 ± 5 W and 60 kick/min separated by 3 min of rest	Blood lactate = 11.4 ± 4.2 mM Epinephrine: 0.75 ± 0.45 nM Norepinephrine: 7.5 ± 5.5 nM	0 h: → 1 h: ~3.0↑ 3 h: ~3.8↑ 5 h: ~2.5↑	0 h: → 1 h: ~1.9↑ 3 h: ~1.9↑ 5 h: ~1.9↑	n/a	0 h: → 1 h: ~2↑ 3 h: ~2.1↑ 5 h: →	0 h: → 1 h: → 3 h: → 5 h: →	0 h: ~2.0↑ 1 h: ~2.0↑ 3 h: ~2.2↑ 5 h: ~2.0↑
Nordsborg *et al.* (2010b)	Untrained (n=8) 44 mL/kg/min	4 x 4 min at 83 ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 10.8 ± 0.5 mM (arm vein)	1+3 h: ~2.0↑	1+3 h: →	n/a	1+3 h: ~2.1↑	n/a	n/a
Nordsborg *et al.* (2010b)	Trained males (n=10) 55 mL/kg/min	4 x 4 min at 85 ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 10.1 ± 0.4 mM (arm vein)	1+3 h: ~2.0↑	1+3 h: →	n/a	1+3 h: ~1.7↑	n/a	n/a
Nordsborg *et al.* (2010b)	Trained males (n=6) 55 mL/kg/min	4 x 4 min at 70 ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 3.1 ± 0.7 mM (arm vein)	1+3 h: ~1.4↑	1+3 h: →	n/a	1+3 h: →	n/a	n/a
Nordsborg *et al.* (2010b)	Untrained (n=6) 44 mL/kg/min	~3 h 20 min at 44 ± 1% VO_{2peak} (exhaustion)	n/a	1 h: ~3.0↑	1 h: ~1.8↑	n/a	1 h: →	n/a	n/a
Nordsborg *et al.* (2010b)	Trained males (n=6) 55 mL/kg/min	~3 h 45 min at 44 ± 1% VO_{2peak} (exhaustion)	n/a	1 h: →	1 h: →	n/a	1 h: →	n/a	n/a
Petersen *et al.* (2005)	Healthy males (n=8) and females (n=7) 51 mL/kg/min	5 min 52 s ± 4 min 27 s at 40% of total work (exhaustion)	Plasma volume: ~3.3-12.3 % ↓ Plasma [K^+]: ↑ from 3.9 (rest) to 4.3 (exercise peak) mmol/L	0 h: ~1.5↑ Mean of 0+3+24 h: ~1.5↑	0 h: ~2.5↑ Mean of 0+3+24 h: ~3.3↑	0 h: ~2.4↑ Mean of 0+3+24 h: ~1.4↑	0 h: → Mean of 0+3+24 h: ~1.1↑	0 h: ~1.7↑ Mean of 0+3+24 h: ~2.0↑	0 h: → Mean of 0+3+24 h: ~1.0↑

To retrieve all published literature on this topic, a search was performed on databases Pubmed, Google Scholar, SportDiscuss, Web of Science, and MEDLINE, using the following keywords: Na+-K+, ATPase, Na-K, pump, mRNA, exercise, muscle. → and ↑ denote no change and increase ($p<0.05$), respectively, compared to baseline.

1.4. Cellular mechanisms regulating Na^+,K^+-ATPase expression and K^+ handling in skeletal muscle

Exercise demands muscle fibres to contract in a coordinated fashion. This process is mediated by action potential (AP) propagation along sarcolemma and down transverse tubules, where activation of voltage-sensors enables release of Ca^{2+} from the SR and resultant initialisation of the excitation-contraction coupling. AP propagation is mediated by Na^+ influx, which causes membrane depolarisation and K^+ efflux. During the repolarisation phase, K^+ is taken up by the fibres, while influx of chloride ions (Cl-) may also participate in this phase (McKenna *et al.*, 2008; Clausen, 2015). Thus, contracting muscle fibres are exposed to constant perturbations in ion homeostasis. In addition to these perturbations, contracting fibres are under a constant redox disequilibrium due to contraction-induced changes in transmural pressure, resulting in episodes of ischaemia (or anoxia) separated by periods with reoxygenation. These oscillations in redox homeostasis create a favourable environment for the production of free radicals (Clanton, 2007; Stoner *et al.*, 2007), classified as molecules that contain one or more unpaired electrons (Powers *et al.*, 2011a). While an imbalance in both ion and redox homeostasis and free radical production have been implicated in the aetiology of muscle fatigue (Allen *et al.*, 2008; Clausen, 2015), involvement of these processes in training adaptation is an emerging area of research in humans. In this section, the role of these processes in regulating K^+ handling and the expression of Na^+,K^+-ATPase isoforms will be discussed. Most of our current knowledge on this topic stems from experiments *in vitro* using cell cultures and animal tissue. These experiments will thus be the centre of the following discussion. However, human studies will be referenced where possible.

1.4.1. Oxygenation and redox balance

The severity of fluctuations in local oxygen levels in contracting muscle fibres may vary with exercise duration and intensity and thus may partly determine the amount of free radicals that are formed in exercising muscles (Clanton, 2007). Considering the role of free radicals in regulating Na^+,K^+-ATPase-isoform expression (Silva & Soares-da-Silva, 2007; Murphy *et al.*, 2008), muscle oxygenation could thus be a critical determinant of exercise-induced increases in Na^+,K^+-ATPase protein abundance. The level of oxygen *per se* may also directly modulate Na^+,K^+-ATPase-isoform expression. For example, in rabbit kidney cells, increasing the oxygen level of the cell bathing solution caused an increase in α_1 (Wendt *et al.*, 1998b) and β_1 (Wendt *et al.*, 1998a; Wendt *et al.*, 1998b; Wendt *et al.*, 2000) mRNA expression independent of ROS. In another experiment using lung tissue of piglets, increases in global Na^+,K^+-ATPase mRNA and protein content were evident after breathing hyperoxic gas (inspired oxygen fraction = 0.96) (Youssef *et al.*, 1999). In contrast, lowering the oxygen perfusion of skeletal muscles in

humans by training in normobaric, systemic hypoxia (Green *et al.*, 1999b) or by living at high (and training at low) altitude (Aughey *et al.*, 2006; Nordsborg *et al.*, 2012) either decreased or did not affect, respectively, both abundance of Na^+,K^+-ATPase isoforms and plasma K^+ concentrations during exercise. Collectively, these results suggest that an increased muscle oxygen level, in contrast to hypoxia, is a potent signal for increases in expression of Na^+,K^+-ATPase isoforms. However, no studies have addressed the role of muscle oxygenation *per se* in exercise-induced regulation of expression of ion transport systems in humans.

1.4.2. Reactive oxygen species

Reactive oxygen species (ROS) is a broad term used to classify oxygen-centred molecules that contain one or more unpaired electrons, but also includes reactive derivates of oxygen such as hydrogenperoxide (H_2O_2). ROS is one of two subcategories of free radicals. The other subcategory is reactive nitrogen species (RNS), which refers to free radicals for which nitrogen is the reactive centre (Powers *et al.*, 2011a). For a thorough inspection of the different types and sources of ROS in skeletal muscle, the reader is referred to the reviews by Powers *et al.* (2011a) and Jackson *et al.* (2007). It is now well-established that chronic increases in ROS levels are involved in the aetiology of many pathological conditions, including type-II diabetes (Bhansali *et al.*, 2017) and peripheral artery disease (Dopheide *et al.*, 2013; Steven *et al.*, 2017). Conversely, transient increases in ROS levels that are rapidly reversible are central to the regulation of normal contractile function (Lamb & Westerblad, 2011), as well as signalling transduction underlying training adaptation (Gundersen, 2011; Powers *et al.*, 2011b). The latter roles of ROS are the focus of the present thesis and thus will be the centre of the following literature review.

Exercise elicits marked increases in ROS levels in skeletal muscle, which can modulate muscle force development in both a time- and dose-dependent manner (Reid *et al.*, 1993). High doses of ROS have been shown to impair myocytic force development by perturbing ion (Ca^{2+} and K^+) homeostasis (Cerbai *et al.*, 1991; Lamb & Westerblad, 2011). Accordingly, antioxidant treatment in humans attenuated exercise-induced increases in arterialised-venous K^+ concentration (Medved *et al.*, 2004a; McKenna *et al.*, 2006), indicating ROS may affect whole-body K^+ homeostasis in exercising humans. Disturbance of K^+ homeostasis due to ROS accumulation is likely mediated via oxidative modifications to, and thereby dysfunction, of K^+ channels and transport systems, such as the Na^+,K^+-ATPase (Kourie, 1998; Lamb & Westerblad, 2011). One type of modification is the formation of disulphide bonds between glutathione and reactive cysteine thiols on amino acid structures (S-glutathionylation), i.e. oxidative damage (Juel, 2014; Juel *et al.*, 2015). Na^+,K^+-ATPase dysfunction induced by severe ROS formation has been demonstrated in cell culture preparations (Shattock &

Matsuura, 1993; Figtree *et al.*, 2009; Liu *et al.*, 2012; Liu *et al.*, 2013), and this has been confirmed by observations of an inverse relationship between degree of glutathionylation of Na^+,K^+-ATPase subunits and Na^+,K^+-ATPase activity in rat skeletal muscles (Juel, 2014) and findings of increased β-subunit glutathionylation coinciding with fatigue during intense exercise in humans (Juel *et al.*, 2015). However, the observation that Na^+,K^+-ATPase is under redox control is not new. The first evidence for such regulation was published in the sixties, where hydrogen peroxide (H_2O_2) treatment markedly depressed Na^+,K^+-ATPase activity in electrical eels (Glynn, 1963). Later, this was confirmed in several other tissues, including the brain (Dobrota *et al.*, 1999), kidney (Boldyrev & Kurella, 1996; Boldyrev & Bulygina, 1997), and myocardium (Kim & Akera, 1987; Kukreja *et al.*, 1990) by actions of the hypochlorous and hyperchlorite anions, hydroxyl radicals, superoxide, and singlet oxygen. Around the same time, it was shown that Na^+,K^+-ATPase activity is inhibited by tert-butyl hydroperoxide at high, but not at low concentrations (Sen *et al.*, 1995), indicating a dose-dependent effect of ROS on Na^+,K^+-ATPase function, which appears similar to that for muscle contractile function highlighted by Lamb and Westerblad (2011). On this basis, tight control of ROS levels appears necessary to preserve K^+ homeostasis and contractile performance of skeletal muscle. Despite this evidence, it remains presently unexplored if ROS are involved in regulating K^+ homeostasis in the exercising musculature of humans.

To counteract oxidative damage during periods of elevated ROS exposure, muscle fibres contain a network of strategically-located ROS scavenging systems, including enzymatic and non-enzymatic antioxidants, defined as substances that either delay or hinder oxidation of a substrate (Powers *et al.*, 2011a). The primary antioxidant enzymes are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). These enzymes catalyse the degradation of highly-reactive ROS into less-reactive molecules. Non-enzymatic antioxidants also provide a significant reservoir for ROS scavenging and this term covers substances such as glutathione, uric acid, bilirubin, biliverdin, and carnosine, among others (Severin *et al.*, 1984; Sen, 1999; Jansen & Daiber, 2012; Fabbrini *et al.*, 2014). The content and function of these scavengers are typically altered by exercise training. In humans, for example, exercise training, regardless of the type being performed (e.g. endurance, resistance, or a combination), has been reported to increase the activity and/or expression of SOD, GPx, glutathione:glutathione disulphide ratio, total antioxidant capacity, and/or decrease oxidative damage in blood, erythrocytes, or skeletal muscles (Hellsten *et al.*, 1996; Leeuwenburgh *et al.*, 1997; Powers *et al.*, 1999; Azizbeigi *et al.*, 2014). Thus, if ROS are involved in regulating muscle K^+ handling in humans, as suggested by Medved *et al.* (2004a) and McKenna *et al.* (2006), an increase in antioxidant function could be a contributing factor underlying the improved skeletal muscle K^+ handling observed after a period of intense training (Nielsen *et*

al., 2004; Gunnarsson *et al.*, 2013). In support of this hypothesis, antioxidant treatment may acutely reverse ROS-induced oxidative inhibition of ion channels and transport systems (Kourie, 1998). Nevertheless, this hypothesis remains currently untested in humans.

In addition to their acute impact on ion handling mechanisms, ROS may be involved in the long-term (chronic) regulation of muscle Na^+,K^+-ATPase expression by exercise training via their actions as signalling transducers for mRNA transcription and protein synthesis (Murphy *et al.*, 2008; Powers *et al.*, 2011b; Petersen *et al.*, 2012). In humans, involvement of ROS in regulating the turnover of mRNAs of importance to ion transport function has been investigated in one study. In this study, Murphy *et al.* (2008) found that intravenous infusion with the multiple ROS scavenger, N-acetylcysteine (NAC), blunted the rise in Na^+-K^+-ATPase α_2-isoform mRNA during the recovery from 45 min of cycling at 71% VO_{2max}. Further, they observed that pre-incubation of rat EDL muscle with NAC abolished the increase in Na^+-K^+-ATPase α_1, α_2 and α_3 mRNA induced by electrical stimulations *in vitro*. Although the impact of NAC on muscle antioxidant status was not determined in this study, it suggests that ROS accumulation may be a critical determinant of exercise-induced increases in mRNA content of catalytic Na^+-K^+-ATPase isoforms in mammalian muscles. In agreement, long-term treatment of kidney cells with H_2O_2 resulted in upregulation of Na^+-K^+-ATPase expression and activity, whereas adding a bolus of the antioxidant apocynin abolished these effects (Silva & Soares-da-Silva, 2007).

In summary, the level of ROS appears to be a critical factor in both the acute and long-term regulation of the capacity to regulate K^+ and Na^+,K^+-ATPase expression in skeletal muscle. However, most research on this topic has been conducted in animal tissue *in vitro* and cell cultures. In the few human studies published to date, the capacity for K^+ handling was not measured over the exercising musculature, limiting conclusions about a role of ROS and antioxidant function in regulating K^+ to a whole-body level. Further, enhancements in both antioxidant function and K^+ handling can be obtained by exercise training. However, no study has examined if improvement in K^+ regulation with exercise training is related to alterations in antioxidant function. Studies are clearly required to clarify the role of ROS and antioxidant function in the adaptive response of skeletal muscle to training specific to K^+ regulation (and Na^+,K^+-ATPase expression) in humans.

1.4.3. Intracellular Na^+

The first evidence to support a role of intracellular Na^+ ($[Na^+]_i$) in mediating increases in the abundance of Na^+-K^+-ATPase subunits stems from studies using cultured rat cardiac and vascular smooth muscle cells published in the early nineties. In these reports, a rise in $[Na^+]_i$ facilitated by culture incubation with aldosterone (Ikeda *et al.*, 1991), thyroid hormone (Kamitani *et al.*, 1992), ouabain (Yamamoto *et al.*, 1993) and veratridine (Yamamoto *et al.*,

1994) induced a 2 to 7-fold increase in the mRNA expression of the Na^+-K^+-ATPase isoforms. A potent role of $[Na^+]_i$ was later confirmed by findings of increased α_1 and β_1 mRNA in rat kidney epithelial cells and astrocytes incubated with ouabain (Hosoi *et al.*, 1997; Muto *et al.*, 2000). At this time, it therefore seemed plausible that $[Na^+]_i$ could be a key initiator of Na^+-K^+-ATPase gene transcription. But Murphy *et al.* (2006a) later observed that incubation with ouabain (2 h), veratridine (30 min), or monensin, a Na^+ ionophore (30 min), abolished the increase in Na^+-K^+-ATPase α_1, α_3, β_1 and β_3 mRNA in rat EDL muscle after intermittent electrical stimulations *in vitro*. They also observed a decline in α_1 and β_2 mRNA content with ouabain and veratridine, whilst all incubations caused a downregulation of β_3. Thus, a chronic high $[Na^+]_i$ might not be beneficial, and could even be detrimental, to the adaptability of the Na^+-K^+-ATPase genes in mammalian skeletal muscle. In contrast, the intermittent electrical stimulations increased the catalytic isoforms (α_1, α_2 and α_3) (Murphy *et al.*, 2006a). This suggests that an oscillatory nature of $[Na^+]_i$ could be a potent stimulus for elevating Na^+-K^+-ATPase mRNA expression. This is in agreement with a 1.4 to 3.4-fold increase in the α_1-, α_2- and α_3-isoform abundance in human muscle in recovery from repeated intense contractions (Nordsborg *et al.*, 2003a; Aughey *et al.*, 2007), which is known to induce drastic fluctuations in $[Na^+]_i$.

1.4.4. Extracellular K^+ and membrane depolarisation

In isolated rat EDL muscle, increasing $[K^+]$ (13 mM) of the muscle bathing solution invoked an increase (160%) in Na^+-K^+-ATPase α_1 mRNA content (Murphy *et al.*, 2006a). This suggests that membrane depolarisation as a result of the increased extracellular $[K^+]$ may be a potent stimulus for increasing muscle α_1 mRNA. In agreement, inhibition of Na^+-K^+-ATPase activity by adding 0.5 to 1.0 mM ouabain, which increases extracellular K^+ (Overgaard *et al.*, 1997), raised α_1 and β_1 mRNA content in astrocytes *in vitro*. Similar effects of depressed Na^+-K^+-ATPase activity have been observed in humans, where increases in α_1 (1.5 fold) and α_2 mRNA (2.5 fold) after fatiguing knee-extensor exercise were inversely correlated with the change in 3-O-MFPase activity from rest to exhaustion ($r = -0.60$ in both cases; $p < 0.05$) (Petersen *et al.*, 2005). Taken together, these observations strongly indicate that extracellular K^+ accumulation and resultant membrane depolarisation positively regulates mRNA levels of catalytic Na^+-K^+-ATPase isoforms in mammalian tissues.

1.4.5. Intracellular Ca^{2+}

In mouse muscle, it has been shown that Ca^{2+} is released from SR in an exercise-intensity dependent manner, which appears to be tightly coupled to activation of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in intact muscle fibres (Aydin *et al.*, 2007). Exercise-induced induction of some Na^+-K^+-ATPase mRNA transcripts is positively associated with

exercise intensity (e.g. isoform α_1, Fig. 1.2). Thus, it is possible that fluctuations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and altered activation of CaMKII could play a role in exercise-induced changes in Na^+-K^+-ATPase-isoform mRNA content in skeletal muscle fibres. This was investigated by Nordsborg *et al.* (2010b). Based on rat muscle incubations *in vitro*, they found the Na^+-K^+-ATPase α_1-isoform to be regulated by Ca^{2+} signalling pathways (CaMK and calcineurin). In another *in vitro* rat experiment, a rise in $[Ca^{2+}]_i$ induced by incubation with the Ca^{2+} ionophore A-23187 elevated the Na^+-K^+-ATPase α_3 mRNA (1.2 fold), but reduced β_1 (0.8 fold). Thus, despite a scarcity of published research, it appears likely that increases in $[Ca^{2+}]_i$ and resultant signalling transduction through either CaMKII, calcineurin, or both, may be involved in exercise-induced regulation of Na^+-K^+-ATPase-isoform mRNA levels in skeletal muscle. However, exercise-induced modulation of the degree of phosphorylation at Thr^{287}, and thus autonomous activity (Rose & Hargreaves, 2003; Rose *et al.*, 2006; Rose *et al.*, 2007), of CaMKII was dissociated from increases in Na^+-K^+-ATPase α_2 and FXYD1 mRNA in human skeletal muscle (Christiansen *et al.*, 2018), indicating signaling via CaMKII is not required for exercise-induced increases in the levels of some Na^+,K^+-ATPase mRNA transcripts in human skeletal muscle. Nevertheless, CaMKII activity has been shown to be upregulated at the onset of exercise, from where it may gradually decrease towards resting level during sustained moderate-intensity exercise (Rose & Hargreaves, 2003). As such, the timing of the muscle biopsy after the end of the exercise session in the latter study may not have been optimal for detecting changes in CaMKII phosphorylation (and activity). Thus, further experiments in humans are warranted to clarify the involvement of altered $[Ca^{2+}]_i$ and activation of Ca^{2+} signaling proteins in mediating Na^+-K^+-ATPase adaptation.

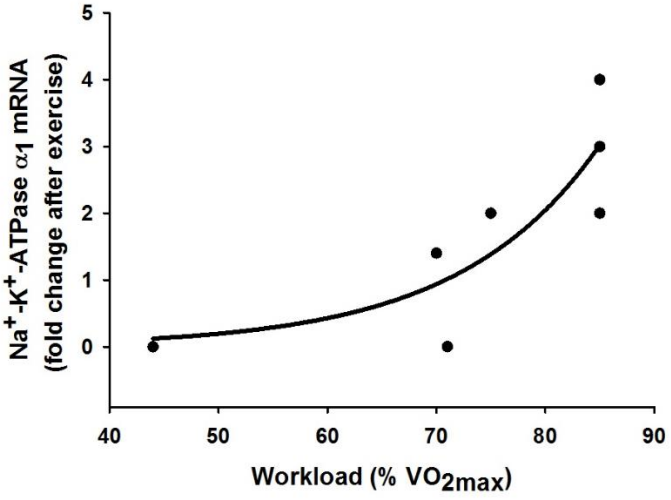

Figure 1.2. Effect of exercise intensity on change in Na^+-K^+-ATPase α_1 mRNA in response to single session of exercise in human skeletal muscle. The figure is based on data from Aughey *et al.* (2007) and Nordsborg *et al.* (2010a) that used similar cohorts (trained humans) and exercise modality (cycling). A single, two-parameter exponential fit [f=a(exp(bx))] provided the strongest relationship between the two factors (r = 0.85).

1.4.6. Energy metabolism and the 5'AMP-activated protein kinase (AMPK)

Amongst the few published human studies (Table 1.2), there is consensus that exercising with a high, compared to a lower, relative intensity yields a more powerful stimulus for induction of ion transport genes (Nordsborg *et al.*, 2003a; Nordsborg *et al.*, 2010b; Rasmussen *et al.*, 2011). This is supported by the positive relationship (r = 0.85) between exercise intensity and fold-increases in muscle Na^+-K^+-ATPase α_1-isoform mRNA in trained humans (Fig. 1.2). Sustained exercise at a high intensity requires a high anaerobic energy turnover, which results in accumulation of H^+ (i.e. a decline in pH) in exercising muscles. In turn, this could impair the function of K^+ and Ca^{2+} transport systems, such as the Na^+-K^+-ATPase and SR Ca^{2+}-ATPase (Kawai *et al.*, 1996; Salonikidis *et al.*, 2000), thereby exacerbating perturbations in ion homeostasis in the exercising musculature. Furthermore, a marked increase in anaerobic glycolytic flux raises the availability of NAD(P)H for ROS production and exacerbate fluctuations in redox homeostasis (Nioka *et al.*, 1998). On this basis, the degree of anaerobic energy turnover appears to be an important determinant of the effects of a given exercise intervention on Na^+-K^+-ATPase-isoform expression. In agreement, decreasing the relative exercise intensity (and thus anaerobic energy turnover) by performing the same exercise session after compared to before a period of intense training attenuated the exercise-induced increase (3 fold) in α_1 mRNA observed before training (Nordsborg *et al.*, 2003a). In addition, exercise has been shown to activate AMPK in an intensity-dependent manner (Chen *et al.*, 2003), whereas increased activity of AMPK has been linked with transcription of both the Na^+-K^+-ATPase β_1 isoform and FXYD1 *in vitro* (Nilsson *et al.*, 2006). Thus, increased AMPK signalling may be another mechanism by which exercise intensity and the resultant altered metabolism may influence the regulation of Na^+-K^+-ATPase-isoform expression. It is presently unknown if AMPK may be part of the signalling cascade underlying increases in abundance of Na^+-K^+-ATPase isoforms in human skeletal muscle.

In summary, increases in expression and function of the Na^+-K^+-ATPase appear to be initiated by transient perturbations in redox, ionic, and metabolic state, whilst $[Na^+]_i$, $[Ca^{2+}]_i$, extracellular $[K^+]$, and ROS may be of particular importance to this regulation. Little is currently understood about how these cellular stressors may affect adaptations in K^+ handling and muscle Na^+-K^+-ATPase abundance in humans. To provide novel insights on this topic, it may thus be useful to develop strategies that can modulate these stressors in human skeletal muscle.

1.5. Training with blood flow restriction

One possibility to augment perturbations in redox, ionic, and metabolic homeostasis in the musculature of humans is to exercise with a reduced muscle blood flow, known as blood flow-restricted training. In previous research, blood flow restriction (BFR) has involved the fixation

of a pneumatic tourniquet (or cuff) at the most proximal part of the limbs and has been applied to various exercise modes, such as walking, cycling and resistance training (e.g. Abe *et al.*, 2006; Abe *et al.*, 2010; Cook *et al.*, 2014). Addition of BFR to exercise has been shown to amplify metabolic by-product accumulation (Inagaki *et al.*, 2011; Sugaya *et al.*, 2011), leading to a more hypoxic and acidic intramuscular environment (Hultman & Sjoholm, 1983; Sundberg & Kaijser, 1992). Thus, BFR could be an effective stimulus to increase the expression of ion transport systems in skeletal muscle by perturbing ion and redox homeostasis. In contrast, too severe an intracellular acidosis may be detrimental to the post-exercise increase in mRNA levels (Nordsborg *et al.*, 2010b) and adaptability of the ion transport systems to exercise training (Thomas *et al.*, 2007). Thus, the severity and duration of BFR could be decisive for its effect on acute and chronic adaptations specific to muscle ion handling.

Superimposition of BFR to exhaustive exercise leads to premature fatigue (Eiken & Bjurstedt, 1987). Although evidence in humans is lacking, this appears to relate to malfunction of ion channels and transport systems (Kourie, 1998; Clausen, 2003; Clanton, 2007). For example, inactivation of the Na^+-K^+-ATPase may occur earlier during exercise with than without BFR due to inhibition of its primary energy pathway (anaerobic glycolysis), which is promoted by intramuscular acidification (Clausen, 2003). BFR exercise may also inactivate the Na^+-K^+-ATPase by increasing the formation of ROS (McKenna *et al.*, 2006; Clanton, 2007). Inhibition of the Na^+-K^+-ATPase leads to an increase in $[Na^+]_i$ and a concomitant rise in $[Ca^{2+}]_i$ via excitation of the Na^+/Ca^{2+} exchanger (Cross *et al.*, 1998). Accumulation of $[Ca^{2+}]_i$ may also be augmented by ROS formation due, in part, to their capability to inhibit the sarcoplasmic reticulum calcium ATPase (SERCA) (Kourie, 1998). Thus, $[Na^+]_i$ and $[Ca^{2+}]_i$ could both be amplified by exercising with BFR. A rise in the inward osmotic pressure gradient accompanying BFR, forcing fluid to accumulate in the intracellular compartment, may further magnify the ionic perturbations during intense exercise, for example, by increasing the intracellular dilution space for K^+ (Sjogaard *et al.*, 1985). In addition, in cardiac myocytes exposed to different levels of hypoxia, mitochondrial ROS was shown to increase in synchrony with the severity of hypoxia (Duranteau *et al.*, 1998). This suggests that muscle ROS accumulation and resultant oxidative damage could be related to the severity of the ischaemic insult invoked by BFR (Clanton, 2007). Further, in humans, post-exercise muscle oxygenation, a regulator of Na^+-K^+-ATPase expression (cf. section on *Oxygenation and redox balance*), has been shown to be augmented by exercising with BFR (Gundermann *et al.*, 2012; Mendonca *et al.*, 2015). Thus, both the degree and duration of the tourniquet pressure may determine the effect of BFR on training-induced adaptations in ion transport function. Exercise with BFR has also been found to increase nitric oxide (NO) bioavailability, as indicated by higher plasma nitrate concentration, in humans (Sundberg *et al.*, 1994). Increased NO levels may counteract

oxidative inhibition of Na^+-K^+-ATPase function by stimulating maximal activity (Juel, 2015). Thus, there are some indices that BFR exercise could acutely enhance Na^+-K^+-ATPase function during exercise in humans.

In summary, application of BFR during exercise may amplify redox, ionic, and metabolic perturbations, as well as ROS accumulation, in skeletal muscle, suggesting it could serve as an excellent model to study the role of these cellular mechanisms in training adaptation specific to ion regulation and Na^+-K^+-ATPase in human skeletal muscle. Furthermore, based on the above evidence, BFR training could be a potent strategy to augment acute and long-term adaptations in Na^+-K^+-ATPase expression and function, and the capacity for K^+ regulation. However, this remains to be investigated in humans.

1.6. Post-exercise cold-water immersion

In many sports, competitions are often scheduled within a few days or even on the same day, allowing insufficient time for athletes to physically recover between activities. For example, reductions in physical performance invoked by prior intense exercise have been observed using both short (1 hour) (Bangsbo *et al.*, 1992) and longer (several days) (Mohr *et al.*, 2016) rest periods between activities. A decrease in physical performance due to prior intense exercise has been linked to increased muscle damage, inflammation, and pain (Barnett, 2006). Strategies that limit or hinder increases in these factors could thus serve as potent ergogenic aids. One of these strategies is immersion of the exercised limbs into cold water (≤15 ºC for 10 to 20 min), known as cold-water immersion (CWI). CWI is now a well-integrated practice in many elite sports. It has been shown to hasten recovery of physical performance, possibly by reducing exercise-induced inflammation, oedema, and pain (Yanagisawa *et al.*, 2003; Barnett, 2006; Bailey *et al.*, 2007; Ascensao *et al.*, 2011; Versey *et al.*, 2013), although the ergogenic effect of CWI could be partly placebo-mediated (Broatch *et al.*, 2014). Given these effects, athletes may be able to train with higher quality or with greater loads by use of CWI during the recovery between consecutive, physically-demanding activities. However, despite an overwhelming scientific support for its use, some studies reported no (Coulange *et al.*, 2006; Higgins *et al.*, 2013; Christensen & Bangsbo, 2016) or even a detrimental (Crowe *et al.*, 2007) impact of CWI on performance recovery.

While most published research on post-exercise CWI has focused on its effects on recovery of physical performance, few studies have addressed its impact on skeletal muscle adaptation to exercise training. In one study, CWI performed after each training session reduced anabolic signalling and adaptations specific to muscle hypertrophy in response to resistance training (Roberts *et al.*, 2015), indicating CWI may retard adaptations to training important for

increases in muscle size and strength. In contrast, regular use of post-exercise CWI was observed to increase muscle capillary density and alter changes in fibre type composition with twelve weeks of resistance training in recreationally-active men (D'Souza *et al.*, 2018). Further, CWI had no impact on increases in mitochondrial content and 2-km cycling time trial performance following a period of sprint-interval training in similarly-trained individuals (Broatch *et al.*, 2017). Thus, effects of CWI on training adaptation clearly depends on the component of muscle function studied and/or the type of training performed. It remains presently unexplored how CWI performed after each training session may affect training-induced adaptations specific to ion regulation, such as the abundance of Na^+-K^+-ATPase isoforms, in human skeletal muscle.

Several studies have shown that CWI may affect both mRNA responses to exercise and those of proteins to training (Versey *et al.*, 2013; Roberts *et al.*, 2015; Broatch *et al.*, 2017). During CWI (8°C), muscle blood perfusion is decreased (Gregson *et al.*, 2011) and non-shivering thermogenesis may occur. These conditions may alter redox and ion balance. In agreement, cold exposure has been demonstrated to increase the production of reactive oxygen species (ROS) (Selman *et al.*, 2002), which may impact ion homeostasis (Kourie, 1998). Further, CWI may elevate blood concentrations of norepinephrine (Gregson *et al.*, 2013), which has been linked to increased oxidative stress (Juel *et al.*, 2015). In the previous sections of this chapter, these stimuli were identified as important for increases in Na^+-K^+-ATPase-isoform expression. On this basis, it may be hypothesised that CWI may be one strategy to augment increases in mRNA content and the protein abundance of Na^+-K^+-ATPase isoforms with exercise training in human skeletal muscle. However, these hypotheses remain to be tested.

1.7. Aims of the thesis

With the use of BFR and CWI to modulate the physiological response to exercise and training, the first two aims of the present thesis were:

- To elucidate the key cellular mechanisms regulating adaptations in the expression of Na^+,K^+-ATPase isoforms and K^+ handling in response to exercise training in human skeletal muscle.
- To examine how the abundance of Na^+,K^+-ATPase isoforms is regulated in type I and II muscle fibres by different types of exercise training in humans.

The third aim of the thesis was to develop a novel method for easy and rapid fibre type identification of single fibres from human skeletal muscle samples.

CHAPTER II: Methodological considerations

In this chapter, the main considerations regarding the experimental, training, and biochemical methods used in the studies described in the present thesis will be highlighted. For an in-depth, chronological description of each method, the reader is referred to the respective papers (I-IV).

2.1. Experimental approach

The rationale underlying the experimental approach of the present thesis is shown in Fig. 2.1. A central aspect of this approach was to differently modulate, and where possible isolate, cellular mechanisms identified as important for adaptations specific to K^+ regulation *in vitro* (Chapter I). This was done by incorporating both primarily aerobic (intensity ≤ VO_{2max}) and highly anaerobic (intensity > VO_{2max}) training modalities with and without additional stimuli, such as blood flow restriction (paper I+II), systemic hypoxia (paper I), antioxidant treatment (paper II), and cold-water immersion (paper III). Furthermore, a number of biochemical (dot blotting, western blotting, real-time PCR, and fluorometric assays), invasive (intravenous antioxidant infusion, femoral arterial and venous catheters, and muscle biopsies), and non-invasive techniques (near-infrared spectroscopy and ultrasound Doppler) were integrated (Table 2.1, next page), and various exercise models used (unilateral cycling and isolated knee-extensor exercise). In addition, the methodological approach to analysing protein abundance and phosphorylation comprised antibody validation using positive/negative controls and recombinant protein, loading of and normalisation to a standard curve on each gel, and assessment of western blot variability. These steps were introduced to minimise the possibility of reproducibility and validity issues (Chapter I). Current methods for fibre type-specific protein analysis are costly, tedious, and time consuming, which dramatically reduces the time available for other aspects of a given project. It was thus also considered how existing methods for fibre type-specific protein analysis may be optimised and further developed (paper

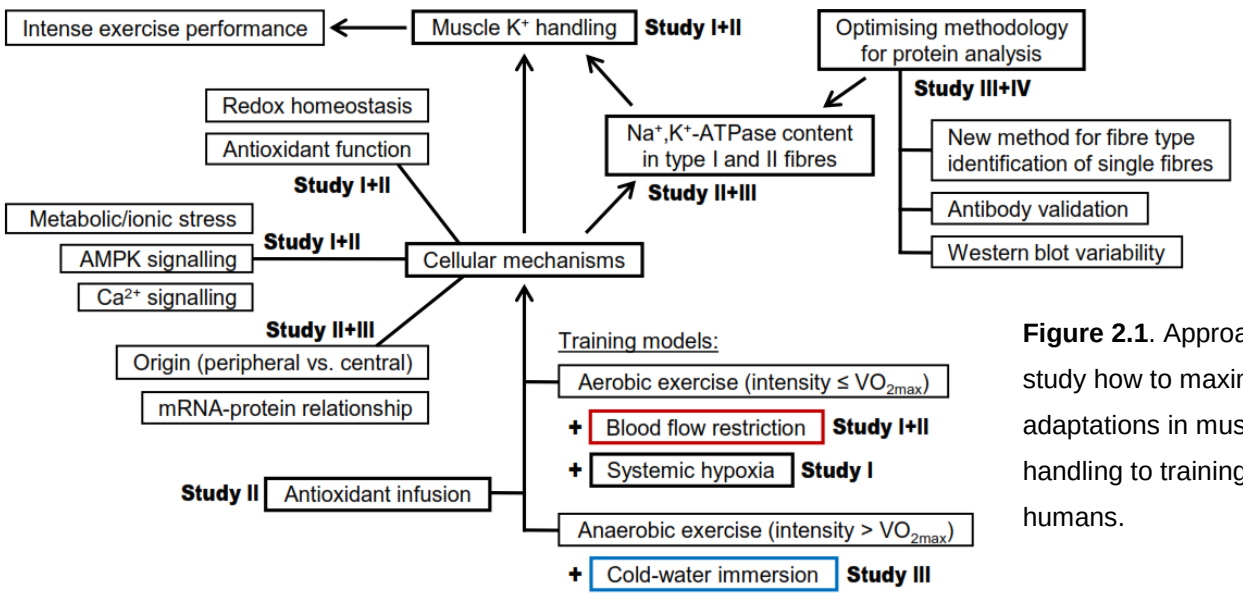

Figure 2.1. Approach to study how to maximise adaptations in muscle K^+ handling to training in humans.

Table 2.1. Invasive, non-invasive, and biochemical methods used in the thesis to measure physiological variables

Physiological variable	Study	Method(s)	Measurement variable(s)
Cellular stressors			
Muscle hypoxia	I	Near-infrared spectroscopy	Deoxygenated haemoglobin
Muscle ions + metabolites	I	Fluorometric assay	Lactate, phosphocreatine, creatine, ATP
Blood/plasma ions	I + II	Arterial and venous catheters	K^+, H^+/pH, lactate
Oxidative stress	I	Single-fibre western blotting, real-time PCR	Heat-shock protein 27 and 70, catalase
Signalling pathways			
AMPK activation	I	Single-fibre western blotting	AMPK and ACC phosphorylation
Ca^{2+} signalling	I	Single-fibre western blotting	CaMKII and phospholamban phosphorylation
mRNA and protein adaptations			
mRNA content	I + III	Real-time PCR	Na^+,K^+-ATPase isoforms
Protein abundance in type I and II fibres	I + II + III + IV	Single-fibre western blotting + dot blotting	Na^+,K^+-ATPase isoforms, AMPK, SERCA.
Functional adaptations			
Antioxidant function	II	Femoral intravenous infusion of N-acetylcysteine	muscle K^+ release
Muscle blood flow	II	Ultrasound Doppler	Femoral arterial blood flow
Systemic K^+ homeostasis	I + II	Arterial and/or venous catheters	Plasma K^+ concentration
Muscle K^+ handling	II	Calculated from blood flow and blood K^+ concentrations	Thigh K^+ release
Single-leg exercise performance	II	Incremental knee-extensor exercise to exhaustion in a Krogh ergometer	Aerobic peak power output

Methods are detailed in paper I to IV of the thesis.

2.2. Subjects

Healthy, young men engaged in team sports, resistance/fitness training, cycling, and/or running at a recreational level several times per week were recruited. Their characteristics are shown in Table 2.2.

Table 2.2. Participants included in studies presented in this thesis (data are means ± SD)

Study	**I**	**II**	**III**	**IV**
Design:	Crossover	Within-subject	Two-group	Within-subject
Number of subjects	8	10	19 (mRNA) and 13 (protein)	1
Age (years)	26 ± 5	25 ± 4	24 ± 6	26
Height (cm)	177.3 ± 7.6	183 ± 6 cm	181 ± 10	181
Body mass (kg)	74.3 ± 7.2	83.6 ± 14	79.5 ± 10.8	79
VO_{2max} *(mL/min/kg)*	57.4 ± 6.2	49.7 ± 5.3	44.6 ± 5.8	52
Training routine	Recreationally active	Recreationally active	Recreationally active	Recreationally active
Country of origin	Australia	Denmark	Australia	Canada

2.3. Development of the blood-flow-restriction protocol

In paper I and II, a reduction in blood flow to exercising muscles was achieved by inflation of a pneumatic tourniquet placed around the proximal portion of the thigh. The cuff was made of nylon with a width of 13 cm (Riester, Jungingen, Germany). Before the onset of experiments for paper I, a BFR protocol was developed to most effectively promote the key cellular mechanisms underlying adaptations in K^+ handling and Na^+,K^+-ATPase content. As described in Chapter I, these mechanisms may be transient perturbations in ion homeostasis, oxygen tension, and ROS accumulation. With basis in existing literature, a hypothetical model of fluctuations in skeletal muscle oxygen tension and ROS production during BFR exercise and subsequent reperfusion rest can be drawn (Fig. 2.2, next page). According to this model, the availability of ROS substrate (i.e. oxygen and NAD(P)H) mainly dictates the amount of ROS that are formed (Clanton, 2007; Stoner *et al.*, 2007). Further, ROS production peaks during the reperfusion phase, where the convective oxygen delivery to tissues is maximal (Granger, 1988; Dhalla *et al.*, 2000; Clanton, 2007; Stoner *et al.*, 2007; Raedschelders *et al.*, 2012). In agreement, in paper II, skeletal muscle blood flow reached its maximum within the first minute after the end of BFR exercise, where the tourniquet was deflated (Fig. 2.3, next page). On this

basis, repeated exercise bouts with BFR separated by few minutes with intact blood flow to exercised muscles may be the most effective BFR strategy to augment perturbations in muscle oxygenation, ROS accumulation, and ionic stress (Chapter I). In addition, exercise bouts with BFR may preferably be several minutes in length to markedly, but only transiently perturb ion (and redox) homeostasis (Gottlieb, 2011), whereas a moderate exercise intensity may be chosen to avoid premature fatigue caused by BFR (see pilot study in paper I).

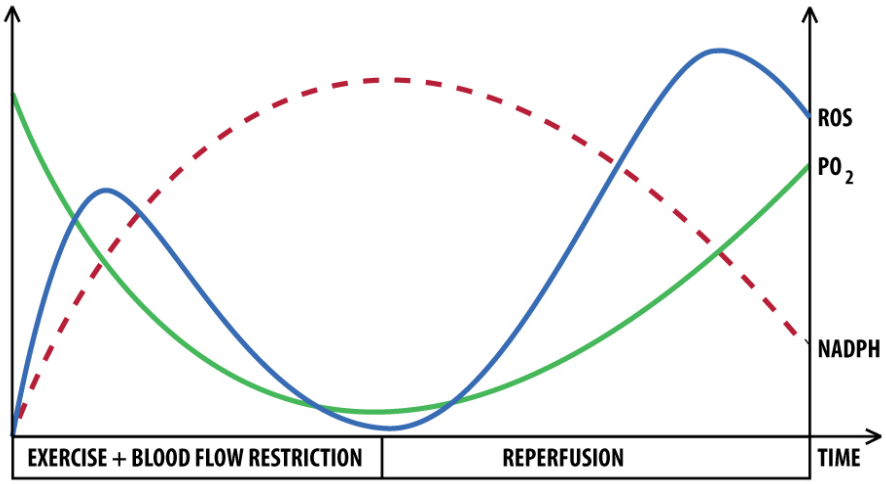

Figure 2.2. Proposed model for fluctuations in reactive oxygen species (ROS) formation, anaerobic glycolytic substrate production for ROS synthesis, and oxygen partial pressure, in skeletal muscle during exercise with blood flow restriction and during the subsequent recovery with intact blood circulation (reperfusion). ROS: blue; PO_2: green, NADPH: hatched red.

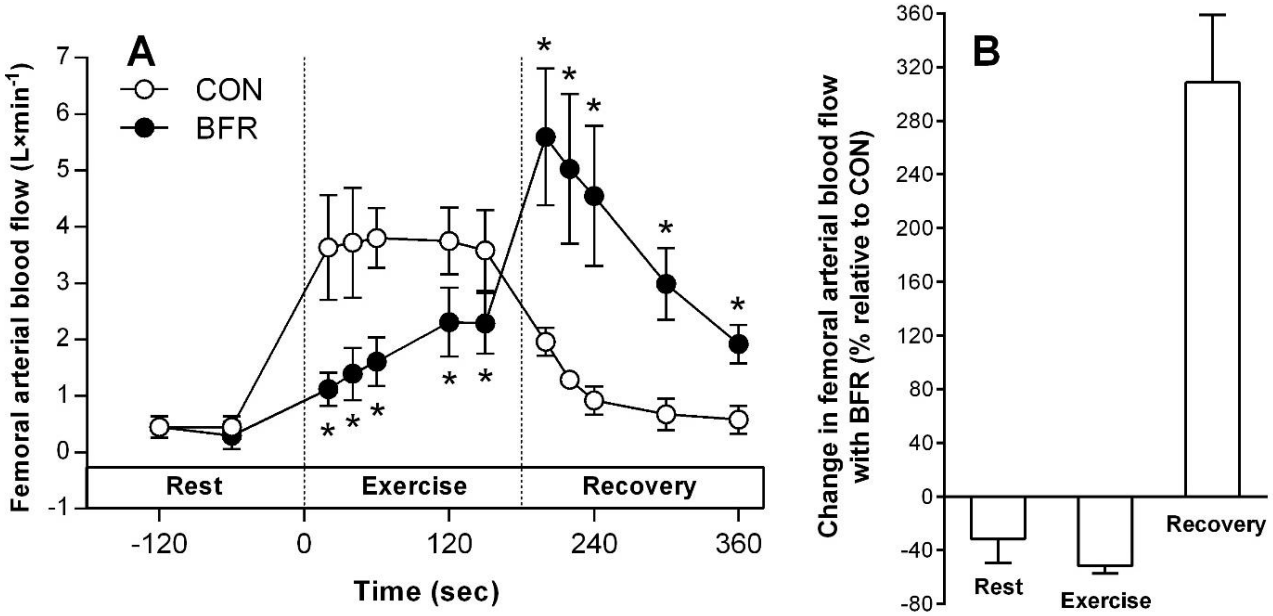

Figure 2.3. Absolute (A) and relative (B) changes in thigh blood flow during and in recovery from moderate-intensity (12 W), single-leg, isolated knee-extensor exercise without (CON) or with blood flow restriction (BFR; ~175 mmHg). Data are expressed as means ± SD.

Given these considerations, the BFR protocol used in the present thesis consisted of three series of 3 x 2-min exercise bouts performed at ~60 to 80% of maximal aerobic power (W_{max}), with 1 and 5 min of recovery between bouts and series, respectively. The tourniquet was inflated 10 s prior to and deflated immediately after each exercise bout. The pressure of the tourniquet was determined in a pilot study, where several exercise sessions were completed with varying degree of BFR (pressure range: 100 to 250 mmHg; n=2 subjects). The highest tolerable pressure, by which the exercise protocol could be completed, was chosen for experiments in paper I and II (~175 mmHg).

2.4. Modulation of physiological stressors in paper I

In paper I, muscle levels of hypoxia, oxidative stress, and ions were manipulated during exercise using blood flow restriction and normobaric, systemic hypoxia. An overview of how the stressors were manipulated is illustrated in Table 2.3.

Table 2.3 Manipulation of key cellular stressors during exercise with blood flow restriction and systemic hypoxia in paper I

	Aerobic exercise (control)	**Aerobic exercise with blood flow restriction**	**Aerobic exercise in systemic hypoxia**
Technical variables			
Absolute intensity	~12 km/h	~12 km/h	~12 km/h
Cuff pressure	No pressure	~175 mmHg	No pressure
Altitude	Sea level	Sea level	~3250 m
Face mask	On	On	On
Pressure cuff	Attached	Attached + inflated	Attached
Physiological variables			
Perceived intensity	Low	High	High
Mechanical stress	Moderate	Moderate	Moderate
Muscle hypoxia	~70%	~90%	~90%
Lactate accumulation	Low	High	High
Plasma K^+ changes	Moderate	High	Moderate
Muscle oxidative stress	Moderate	High	Moderate

In paper I, muscle hypoxia (as assessed by near-infrared spectroscopy) during exercise was matched between exercise sessions with BFR and systemic hypoxia (Table 2.3). This was achieved by comparing measurements of muscle hypoxia across exercise sessions with BFR and at different simulated altitudes (~2000 to ~3700 m; n=2). Based on the comparisons, an inspired oxygen fraction of 14%, corresponding to an altitude of ~3250 m, was used in the

hypoxic session to match the level of muscle hypoxia during the session with BFR. To increase the likelihood of matching of muscle hypoxia between the sessions, each subject performed a familiarisation session consisting of three bouts with BFR (mean pressure: ~175 mmHg) and three bouts in systemic hypoxia (F_iO_2 = 14%) with continuous assessment of muscle hypoxia (paper I). Based on data obtained from this session, the inspired oxygen fraction to be used in the main session with systemic hypoxia was further adjusted on an individual basis.

2.5. Unilateral training model

In paper II, a within-subject, matched-work training model was used, where one leg trained with and the other leg without BFR simultaneously. This design allowed us to reduce biological variability and improve the statistical power for the number of subjects included (MacInnis *et al.*, 2017), and to study peripheral mechanisms in isolation from systemic factors.

Matching of work produced by a leg training with BFR with that of the contralateral leg training without BFR was a challenging task, because BFR markedly increased perceived intensity, as shown in Fig. 2.4. Thus, to increase the likelihood of matching the work generated by the legs during training, force-sensor insoles were inserted in the cycling shoes. This enabled visual feedback in real-time about the force produced during each pedal rotation via wireless transmission of force data to a 10-inch display (iPad Mini 2, Apple Inc., California, USA), which was attached to the cycle rail (see illustration in Fig 2.5A; next page). Using this setup, the work produced by each leg during training in paper II was successfully matched, as evident from Fig. 2.5B. The tourniquet pressure was controlled during training sessions by manometers placed on the cycle rail (Fig. 2.5A). Validation of force sensor insoles (Pedoped, novel GmbH, Münich, Germany) was performed against a traditional force plate (Kistler, Sindelfingen, Germany) by comparing the force curve created by application of an external load, as illustrated in Fig. 2.6. The error margin between the force curves of the insoles and the force plate was ≤ 10%.

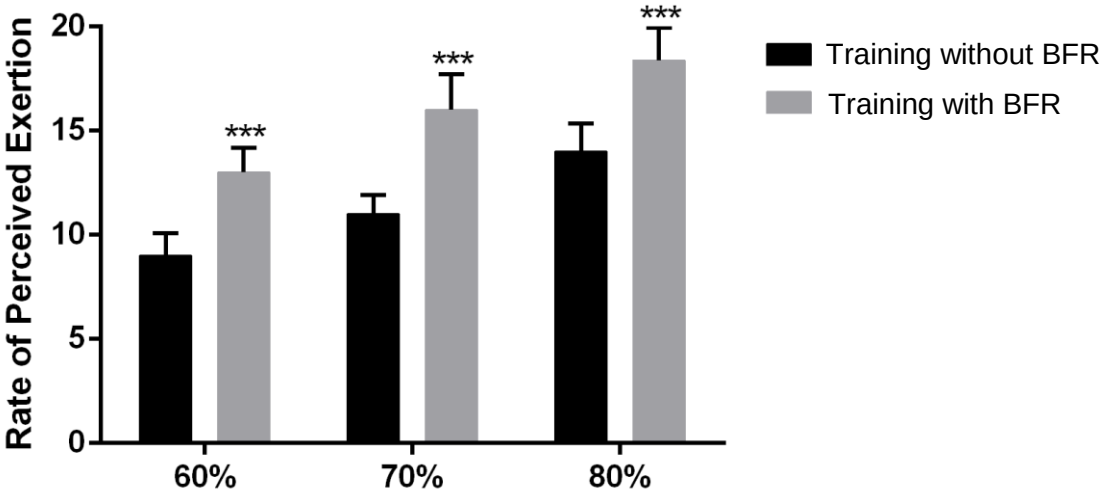

Figure 2.4. Mean subjective rate of perceived exertion for the ten subjects included in paper II at training intensities in percent of maximal aerobic power (%) during the familiarisation period, where both legs trained without blood flow restriction (BFR; black bars; n = 12), and during the intervention period, where one leg trained with BFR (grey bars; n = 18).

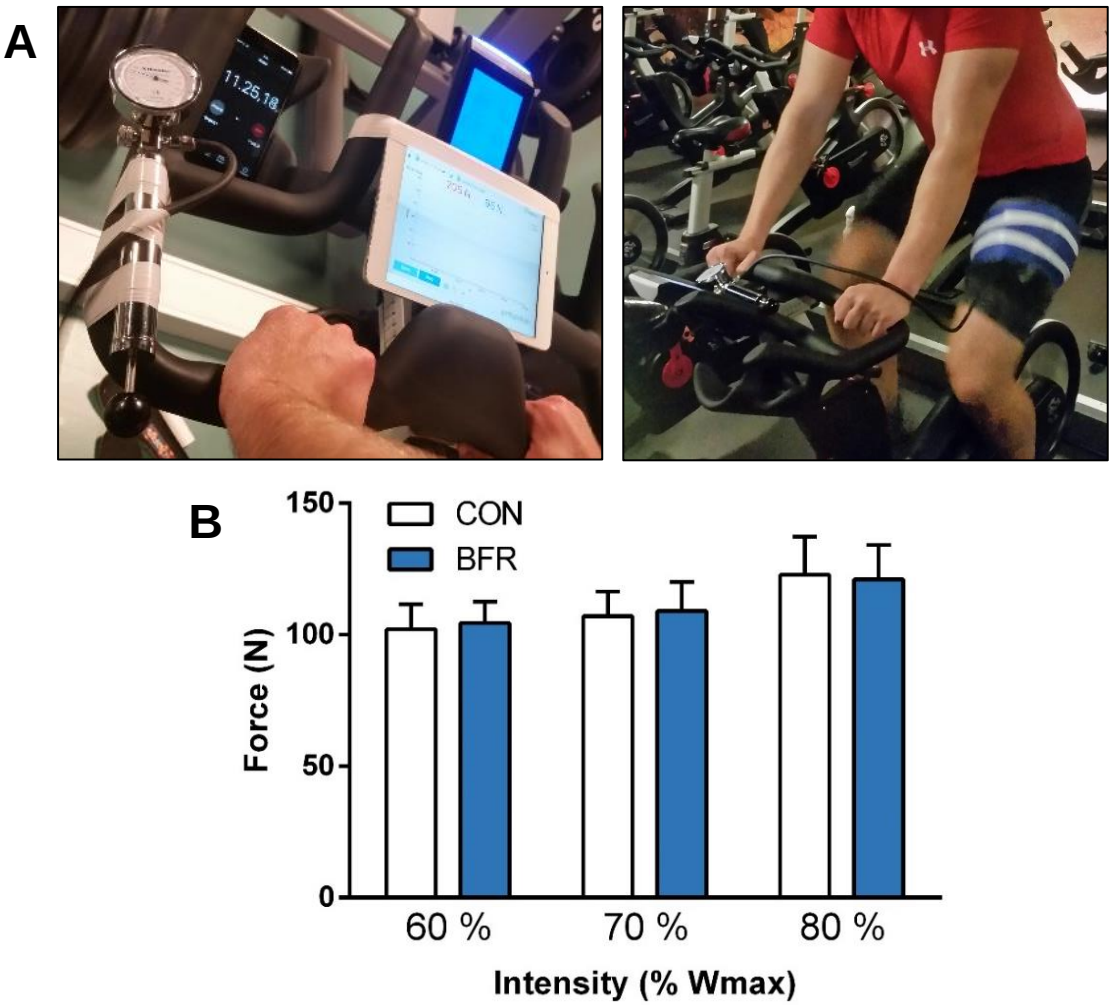

Figure 2.5. A) An illustration of the equipment setup in a training session from paper II. B) Force output of the leg training with (blue bars) and the contralateral leg training without BFR (white bars). No differences were observed in work produced by legs during training ($p<0.05$).

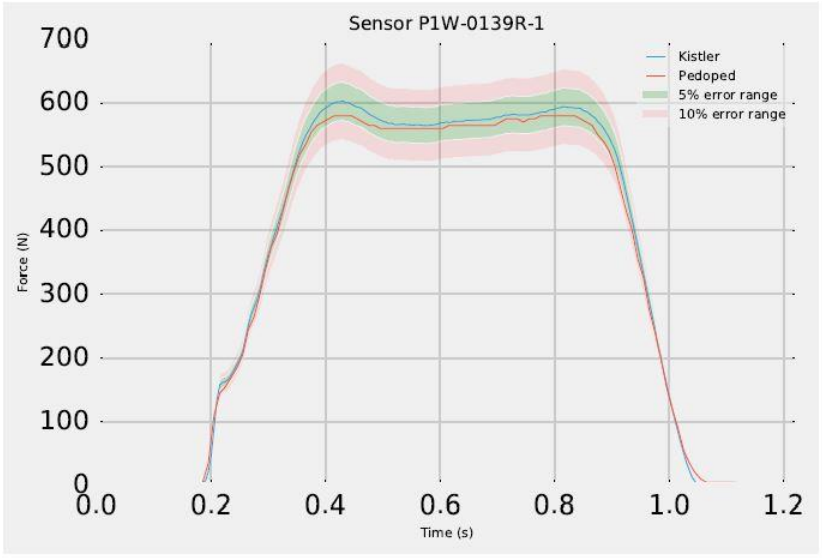

Figure 2.6. An example of the validation of a force-sensor insole in paper II. Pedoped = Insoles, Kistler = force plate. 5% (green) and 10% (red) error margins are illustrated.

2.6. Experimental model in paper II

The single-leg knee-extensor exercise model introduced by Andersen and Saltin (1985) was used in experiments for paper II to measure single-leg performance and K^+ release from exercising muscles under intravenous infusion of antioxidant (N-acetylcysteine) and placebo (saline). After familiarisation to this model, most (90%) of the work and energy turnover during exercise is limited to the quadriceps muscles (Andersen & Saltin, 1985; Gonzalez-Alonso *et al.*, 2000). This is a primary muscle group required in cycling (da Silva *et al.*, 2016), which was the training mode used in paper II. Thus, this model was well-suited to examine local adaptations in the musculature to the training period. In vivo measurement of K^+ concentrations in blood draining exercising muscles was permitted by insertion of catheters in the femoral artery and vein of both legs, whereas femoral artery blood flow was assessed using an ultrasound probe, as illustrated in Fig. 2.7. Together with the use of the muscle biopsy technique, this setup allowed us to evaluate the association between functional and protein adaptations to training with and without BFR in the same individual. Ultrasound Doppler was preferred over the thermodilution technique to assess blood flow, because with the latter method the many measurements of flow in our protocol would have increased the risk of hemodilution. Furthermore, ultrasound Doppler has the advantages of being non-invasive and allows continuous measurement of flow (high temporal resolution) (Gliemann *et al.*, 2018), which was essential in order to track the flow kinetics at onset of exercise and the subsequent recovery in our experiment.

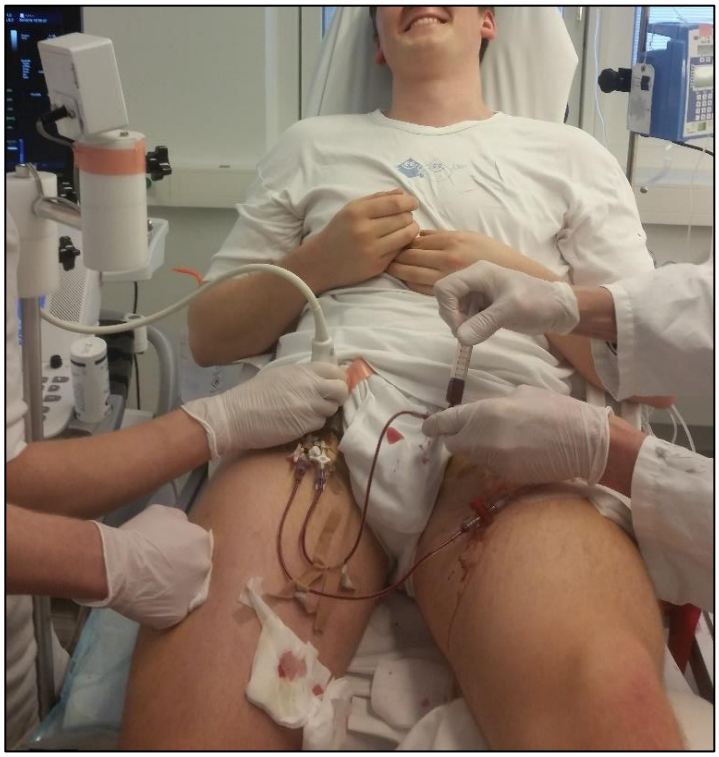

Figure 2.7. Example of the experimental setup used in paper II that enabled in vivo measurement of muscle K^+ release and blood flow during and in the recovery from exercise.

2.7. Antioxidant infusion

The effects of antioxidant infusion on thigh K^+ release was studied before and after training in both legs in paper II. The main purpose of this design was to elucidate if altered antioxidant function with training (with and without BFR) plays a role in regulating the capacity of skeletal muscle for K^+ handling in humans. The antioxidant N-acetylcysteine (NAC) has been shown to circumvent fatiguing exercise-induced reductions in Na^+,K^+-ATPase activity (McKenna *et al.*, 2006) and to raise the concentration of glutathione (Medved *et al.*, 2004b), which serves as buffer for ROS, in human skeletal muscle. NAC has also been used in cell studies to attenuate ROS accumulation caused by ischaemia-reperfusion (Dhalla *et al.*, 2000). Thus, at the time of initialising experiments for paper II, NAC was preferred to study if altered antioxidant function with training would affect K^+ regulation in the musculature.

2.8. Cold-water immersion

Post-exercise cold-water immersion (CWI; 10 minutes in 10°C water up to the umbilicus) was used as an alternative approach to manipulate redox and ion homeostasis in the musculature during training. CWI lowers muscle temperature and raises the hydrostatic pressure, causing a decrease in muscle blood perfusion (reduced femoral artery conductance by 55%) (Gregson *et al.*, 2013; Mawhinney *et al.*, 2017), which may affect redox homeostasis. Non-shivering thermogenesis may also occur as a consequence of lowered tissue temperature, and this has been shown to involve perturbations in muscle $[Ca^{2+}]_i$ (Nowack *et al.*, 2017), a stimulus for increases in Na^+,K^+-ATPase α-subunit expression (Nordsborg *et al.*, 2010a). Elucidating the effects of CWI on training-induced adaptations specific to muscle K^+ regulation was also of great interest at the time of initialising the present thesis, because CWI was (and still is) extensively and widely used as a recovery strategy by elite athletes engaged in many different sports. The cold-water immersion procedure used in the present thesis is illustrated in Figure 2.8.

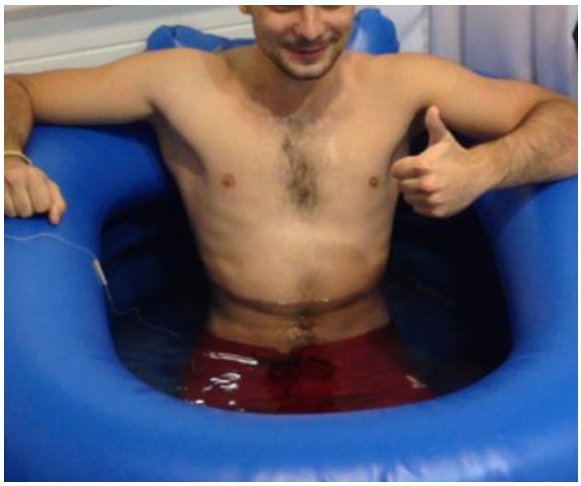

Fig. 2.8. Illustration of the cold-water immersion procedure used in one of the experiments included in this thesis. The water temperature was 10°C and the subjects were immersed up to the umbilicus.

2.9. Measurement of protein abundance in type I and II muscle fibres

Five decades ago, Albers *et al.* (1968) discovered that the cardiac glycoside, ouabain, binds specifically to the Na^+,K^+-ATPase by an ATP-dependent process that is stimulated by Na^+ and inhibited by K^+. Later, it was shown that this binding is saturable, stoichiometric and reversible (Jorgensen & Skou, 1971). These discoveries paved the way for developing a method to quantify Na^+,K^+-ATPase content in rat skeletal muscle preparations *in vitro* by means of radioactively labelled digitalis glycosides, the vanadate-facilitated [^{3}H]-ouabain binding technique (Clausen & Hansen, 1974). This method was later modified to enable quantification of Na^+,K^+-ATPase content in skeletal muscle preparations from humans (Norgaard *et al.*, 1984). Ouabain binds the catalytic α-subunit of the Na^+,K^+-ATPase in human muscle (Rhee & Hokin, 1979), and as such is a measure of total content of Na^+,K^+-ATPase catalytic isoforms in this tissue. Accordingly, there appears to be a positive exponential relationship between training-induced increases in [^{3}H]-ouabain binding and those of α_1 (r^2=0.997) and α_2 (r^2=0.999) protein abundance measured in the same muscle samples by western blotting, as shown in Fig 2.9 (*n* = 4 studies). Whilst the [^{3}H]-ouabain binding technique enables quantitative assessment of Na^+,K^+-ATPase α-subunit content in human muscle, it does not distinguish between isoforms. However, this is important when interpreting effects of interventions such as training and nutrition on muscle function, because these isoforms are functionally different, both in terms of their ion-transport properties (Radzyukevich *et al.*, 2004; Kristensen & Juel, 2010a; Radzyukevich *et al.*, 2013; Manoharan *et al.*, 2015) and signalling actions (Tian *et al.*, 2001; Zhang *et al.*, 2008; Despa *et al.*, 2012; Liu *et al.*, 2013; Mavrogonatou *et al.*, 2015; Madan *et al.*, 2016). With the use of western blotting, relative changes in protein abundances can be measured, highlighting the suitability of this method for the current thesis. Further, western blotting enables post-translational modification of proteins to be studied, which was used in the thesis to (indirectly) examine activation of signalling pathways (paper I) and FXYD1 (paper II) in different fibre types. Limitations and new discoveries in methodology for fibre type-specific protein analysis are discussed in more detail in Chapter III.

In paper III, the abundance of Na^+,K^+-ATPase isoforms was determined in individual type I and II fibres using single-fibre western blotting according to the method described by Murphy (2011a). This method enables classification of fibre type based on myosin heavy chain (or Ca^{2+}-ATPase) presence simultaneously with measuring abundance of proteins of interest in the same fibre segments, thereby reducing time required for sample preparation compared to previous techniques, such as the myofibrillar ATPase-staining method (Essen *et al.*, 1975; Tannerstedt *et al.*, 2009). In paper IV, a novel method for fibre type-specific protein analysis was developed that adopts dot blotting for fibre type identification of fibre segments, before

pooling fibres of the same type for western blot analysis. As highlighted in Chapter III, this method is unprecedented fast and easy to perform, and thus, was used in papers II and III for fibre-type-specific protein analyses. The idea for this method was conceived during experiments for paper III, hence the use in this paper of the older method for fibre type-specific analysis (Murphy, 2011a).

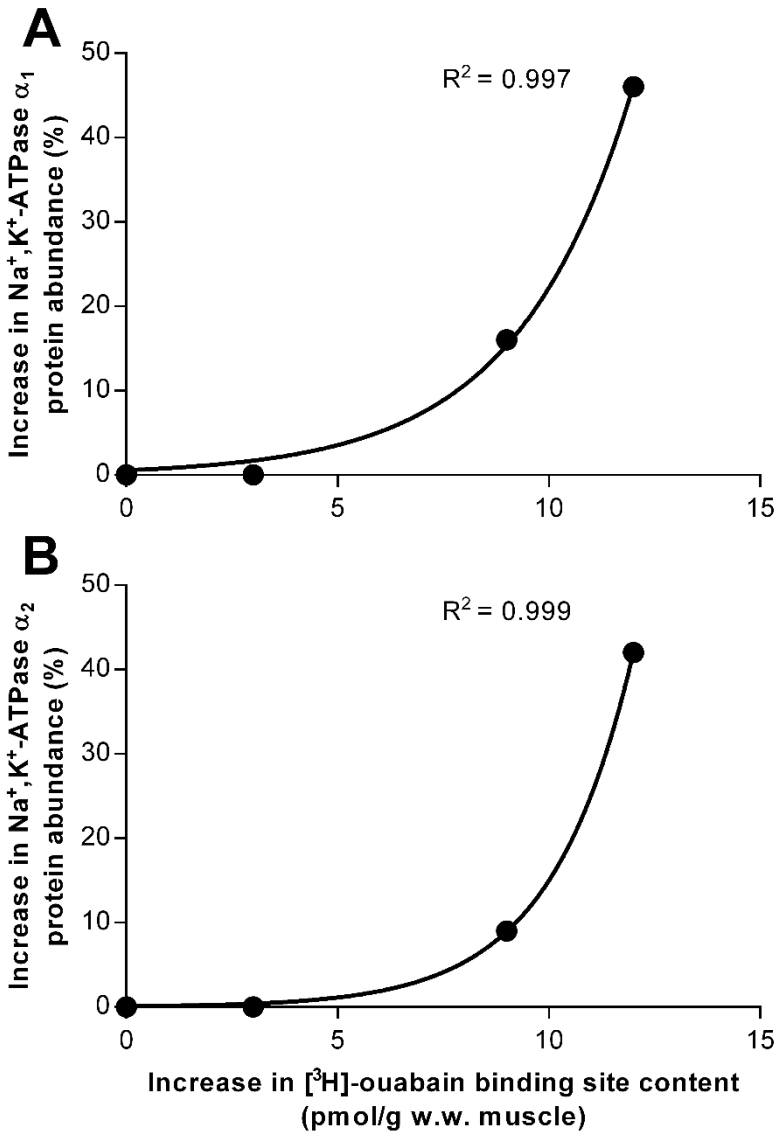

Fig. 2.9. The relationship between pre-training Na^+,K^+-ATPase content (as assessed by [^{3}H]-ouabain binding) and training-induced changes in Na^+,K^+-ATPase α_1 (A) and α_2 (B) isoform protein abundance in human skeletal muscle (Green *et al.*, 2004; Aughey *et al.*, 2007; Green *et al.*, 2008; Perry *et al.*, 2016).

CHAPTER III: Results & General Discussion

In this chapter, the main findings from paper I-IV of particular relevance to the present thesis will be discussed. The discussion is divided into three major sections, each addressing one of the three aims of the thesis. In the first section, key mechanisms regulating the abundance of Na^+,K^+-ATPase isoforms and the capacity for K^+ handling in human skeletal muscle will be discussed, followed by a discussion in section two of fibre-type-specific regulation of Na^+,K^+-ATPase isoforms by different types of training. In the third section, the validity of the dot botting method for fibre type identification of single muscle fibres will be evaluated.

3.1. Mechanisms regulating abundance of Na^+,K^+-ATPase isoforms and the capacity for K^+ handling in human skeletal muscle

The following discussion deals with the main outcomes related to the first aim of the thesis. Based on the literature review (Chapter I), we focused our attention on the following cellular stressors and signalling pathways: Tissue oxygenation (hypoxia/hyperoxia), oxidative damage, ion perturbations, lactate, AMPK and CaMKII signalling, and mRNA expression.

3.1.1. Muscle hypoxia

In paper I, recreational athletes (VO_{2max} = 57 mL/kg/min) completed three interval sessions, in a randomised order, consisting of three sets of three 2-min running bouts. In the first session, intervals were performed with BFR, whereas intervals were executed either in normobaric, systemic hypoxia (~3250 m) or without additional stimuli (control) in the second and third session, respectively. By ensuring the level of muscle hypoxia (deoxygenation assessed by NIRS) was matched between sessions with BFR and systemic hypoxia (Fig. 3.1), we examined whether hypoxia *per se* is important for the adaptive response to exercise in humans. A novel finding was that BFR augmented exercise-induced increases in markers of oxidative stress, AMPK downstream signalling (mainly in type I fibres), and FXYD1 mRNA content. In contrast, exercise-induced responses in Na^+,K^+-ATPase mRNA transcripts and AMPK downstream signalling were not amplified by systemic hypoxia compared to normoxia, despite higher level of muscle hypoxia in the former condition (Fig. 3.1). Collectively, these results demonstrate that the level of hypoxia is not a critical determinant of the acute molecular response underlying adaptations in Na^+,K^+-ATPase expression and K^+ regulation to exercise in human skeletal muscle. Because hypoxia is a central component of the muscle response to BFR exercise and is proposed to be a key factor in training adaptation (Hoppeler & Vogt, 2001; Vogt *et al.*, 2001; Ponsot *et al.*, 2006; Zoll *et al.*, 2006), this observation may seem surprising. But it is in good agreement with, and could partly explain, the lack of a beneficial effect of altitude training concepts, such as the live-high train-low or normobaric hypoxic

interventions, on training-induced adaptations in Na^+,K^+-ATPase function, isoform abundance, and systemic K^+ handling during exercise in recreational athletes (Green *et al.*, 1999b; Sandiford *et al.*, 2004; Aughey *et al.*, 2005; Aughey *et al.*, 2006; Nordsborg *et al.*, 2012).

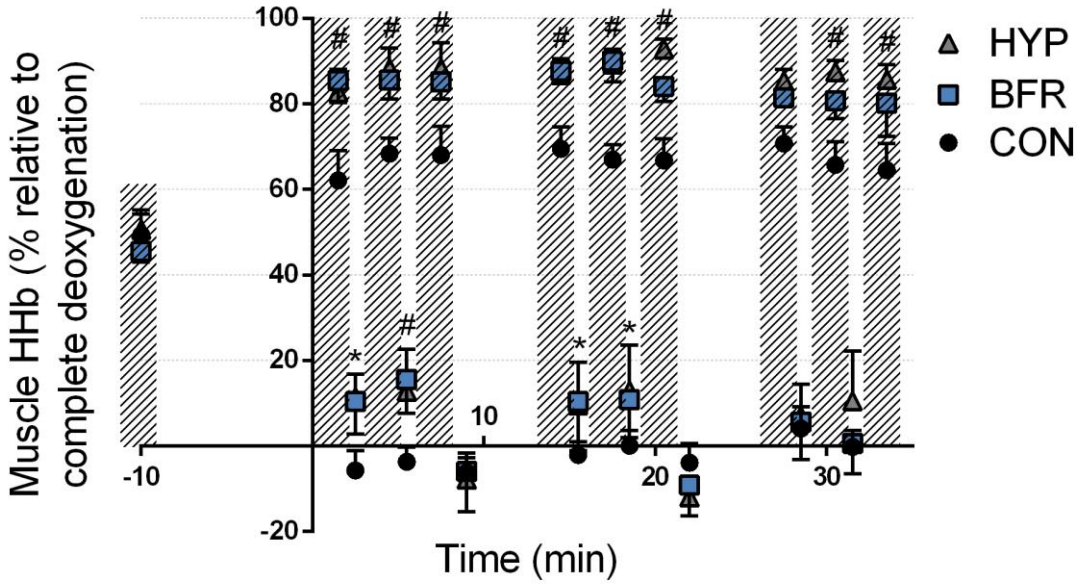

Figure 3.1. Level of hypoxia (deoxygenated haemoglobin, HHb) as assessed by near-infrared spectroscopy in human skeletal muscle during exercise with blood flow restriction (BFR), systemic hypoxia (HYP), and in normoxia (control, CON). Hashed bars represent exercise bouts. #p<0.05, BFR and HYP different from CON; *p<0.05, BFR different from CON; n=8 in all conditions.

3.1.2. Muscle oxygenation and oxidative damage

In contrast to hypoxia, increased tissue oxygenation has been found to increase both α_1 (Wendt *et al.*, 1998b) and β_1 (Wendt *et al.*, 1998a; Wendt *et al.*, 1998b; Wendt *et al.*, 2000) isoform expression, as well as global Na^+,K^+-ATPase mRNA and protein content different cell types *in vitro* (Youssef *et al.*, 1999). In extension of these results, we observed that training with BFR increased the abundance of several Na^+,K^+-ATPase isoforms in type I (β_1), type II (α_1), or both muscle fibre types (FXYD1) in humans (paper II), consistent with the stimulating effect of BFR on muscle oxygen perfusion (>3 fold increase) in recovery from exercise (as assessed by ultrasound Doppler; Fig. 2.3, chapter II). Increased oxygenation could facilitate transcription of Na^+,K^+-ATPase isoforms (Wendt *et al.*, 1998a; Wendt *et al.*, 1998b; Wendt *et al.*, 2000) via activating binding of the transcription factors specificity protein 1 (Sp1) and/or 3 (Sp3) to promoter regions on the mRNA transcripts (Li & Langhans, 2015). The intermittent nature of the training protocol with BFR may also have been important for increasing Na^+,K^+-ATPase-isoform abundance in paper II, because drastic perturbations in redox state effectively increases ROS levels (Simpson & Lucchesi, 1987; Baines *et al.*, 1997; Tritto *et al.*, 1997; Dhalla *et al.*, 2000; Kleikers *et al.*, 2012; Raedschelders *et al.*, 2012), which has been

linked with upregulation of Na^+,K^+-ATPase-isoform expression (Silva & Soares-da-Silva, 2007; Murphy *et al.*, 2008), whereas in paper II the effect of N-acetylcysteine infusion on thigh K^+ release was attenuated in the leg that trained with BFR. In extension of these observations, we found that a single interval exercise session with BFR promoted markers of muscle oxidative damage (a resultant effect of pronounced increases in ROS/RNS levels), whereas several weeks of BFR training increased expression of FXYD1 in type I and II fibres, α_1 in type II fibres, and β_1 in type I fibres (paper II). Further, these changes in protein abundance occurred concomitant with an improved K^+ handling in the exercising musculature, reflected by a decline in net thigh K^+ release during isolated work with the quadriceps muscle (Fig. 3.2; paper II). Together, these results indicate that augmenting oxidative stress during training sessions may be an important stimulus for training-induced enhancements in skeletal muscle Na^+,K^+-ATPase-isoform content and its capacity for K^+ regulation in humans. This conclusion is underlined by observations that oxidative damage to ion channels and transport systems, such as the Na^+,K^+-ATPase, exacerbates disturbances in ion homeostasis (Kourie, 1998; Bibert *et al.*, 2011; Liu *et al.*, 2013), which has been coupled to elevated expression of Na^+,K^+-ATPase isoforms (Murphy *et al.*, 2006a; Nordsborg *et al.*, 2010a). In agreement, greater changes in venous blood K^+ concentration were observed during the exercise session with BFR compared to systemic hypoxia and control (paper I), indicating BFR exercise causes pronounced perturbations in ion homeostasis.

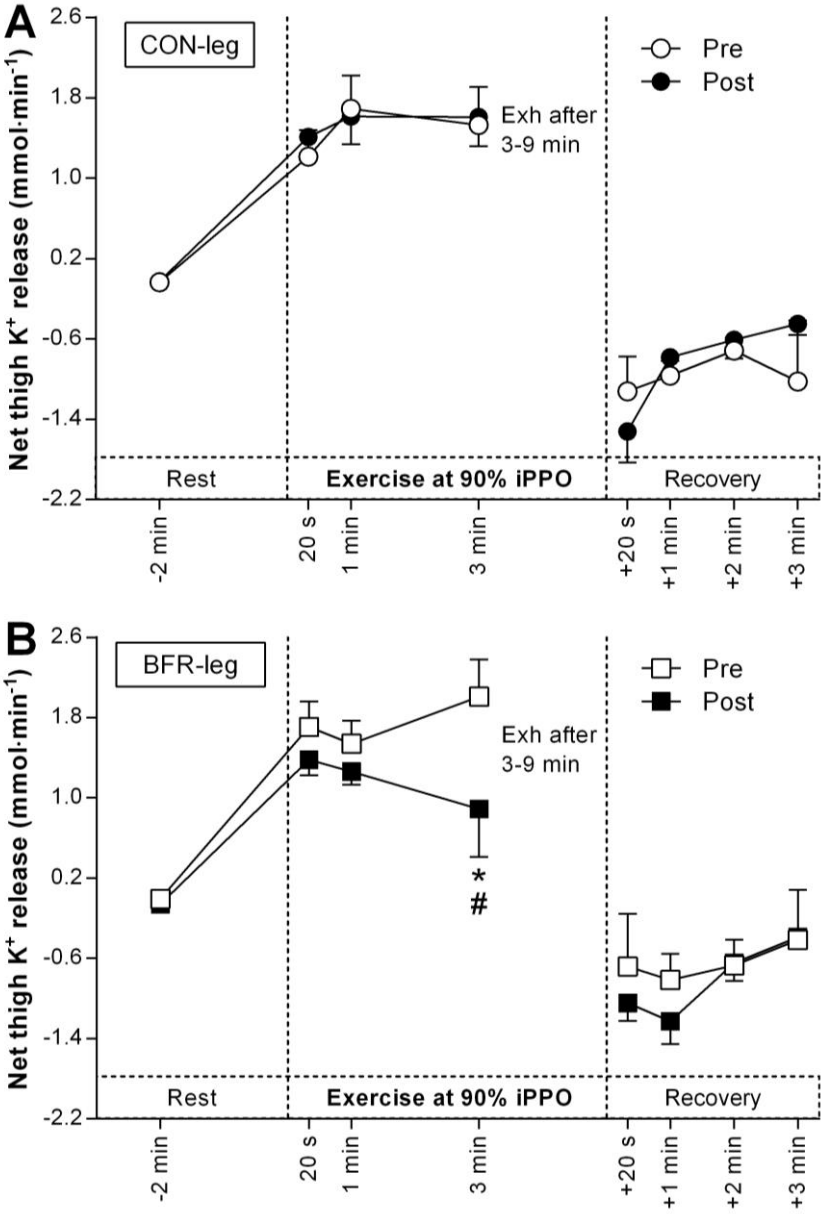

Figure 3.2. Net thigh K^+ release during and in the recovery from isolated exercise with the quadriceps muscle at 90% of aerobic peak power output (iPPO) before (Pre) and after (Post) six weeks of training with one leg using blood flow restriction (BFR-leg) and with the contra-lateral leg without BFR (CON-leg). Exhaustion (Exh) was reached after 3 to 9 minutes of exercise. *p<0.05, different from Pre; #p<0.05, different from CON-leg after the training period.

3.1.3. Muscle lactate

A great anaerobic energy turnover during exercise leads to marked increases in intramuscular H^+ concentration (i.e. low pH value), which can impair the function of K^+ and Ca^{2+} transport systems, such as the Na^+-K^+-ATPase and SR Ca^{2+}-ATPase (Kawai *et al.*, 1996; Salonikidis *et al.*, 2000). Further, an increased anaerobic glycolytic flux has been found to facilitate increases in levels of substrate for ROS production (Nioka *et al.*, 1998), which could promote oxidative damage and thus exacerbated ion perturbations. On this basis, anaerobic ATP production could be an important determinant of the effects of a given training intervention on muscle Na^+-K^+-ATPase-isoform expression and K^+ regulation. In addition, the concentration of lactate, a surrogate marker of anaerobic ATP consumption, has been shown to regulate the expression of several mRNA transcripts involved in various cellular functions, including PGC-

1α, a regulator of mitochondrial content (Kitaoka *et al.*, 2016). While being a by-product of the systematic manipulation of muscle hypoxia by BFR and systemic hypoxia, our experimental setup in paper I allowed us to examine whether lactate concentration could be a regulator of the acute molecular response specific to ion regulation in response to exercise with BFR (Sundberg, 1994; Lundberg *et al.*, 2002) and systemic hypoxia (Richardson *et al.*, 1998). Exercise-induced accumulation of muscle lactate was consistent between exercise sessions with BFR and systemic hypoxia, which was higher than control (paper I). However, changes in the levels of Na^+-K^+-ATPase transcripts were not different after sessions in systemic hypoxia or control. Only BFR augmented exercise-induced increases in FXYD1 mRNA content. Thus, our results suggest that muscle lactate concentration does not play a role in regulating exercise-induced adaptations in mRNA expression of Na^+-K^+-ATPase isoforms in human skeletal muscle. It is important to stress that this observation is limited to the mRNA level. Further research is required to clarify if lactate accumulation during training sessions is an important determinant of (long-term) adaptations in the function and content of the Na^+-K^+-ATPase, and ultimately of improvements in muscle K^+ regulation, in humans.

3.1.4. 5'AMP-activated protein kinase (AMPK)

In paper I, the increase in FXYD1 mRNA with BFR exercise was temporally associated with elevated AMPK downstream signalling, reflected by higher ACC phosphorylation in type I muscle fibres (Fig. 3.3, next page). This observation is consistent with findings *in vitro* that AMPK is involved in the transcription of the FXYD1 transcript (Nilsson *et al.*, 2006). Further, FXYD1 protein abundance increased in both type I and II muscle fibres after training with BFR (paper II). Thus, while a role of AMPK in the acute regulation of Na^+-K^+-ATPase function is well-described (Alves *et al.*, 2010; Ingwersen *et al.*, 2011; Benziane *et al.*, 2012; Lang & Foller, 2014), we provide an indication of involvement of AMPK also in mediating adaptations in expression of FXYD1 in human muscle. The selective increase in ACC phosphorylation in type I fibres may be due to altered metabolism in this fibre type invoked by BFR (Greenhaff *et al.*, 1993). Elevated ACC phosphorylation with BFR exercise may have occurred secondary to an increase in ROS production, which is known to activate AMPK, since BFR exercise increased markers of oxidative damage (paper I) and ischemia-reperfusion has been shown to augment ROS levels (Simpson & Lucchesi, 1987; Baines *et al.*, 1997; Vanden Hoek *et al.*, 1997; Dhalla *et al.*, 2000; Stoner *et al.*, 2007; Kleikers *et al.*, 2012; Raedschelders *et al.*, 2012). A rise in AMP:ATP ratio with BFR exercise could also have contributed to the increase in ACC phosphorylation in paper I (Sundberg, 1994; Friedrichsen *et al.*, 2013; Combes *et al.*, 2015). It should be noted that we examined a correlative, and not a causative, association between ACC phosphorylation and FXYD1. Further, although phosphorylation of ACC strongly reflects AMPK activity (Wojtaszewski *et al.*, 2000), it may have been elevated by factors other than

AMPK. Thus, based on the current findings alone, we are unable to unequivocally state that AMPK is involved in regulating FXYD1 expression in human muscle. Further studies are warranted to determine the relationship between exercise-stimulated changes in muscle AMPK activity and FXYD1 abundance in humans.

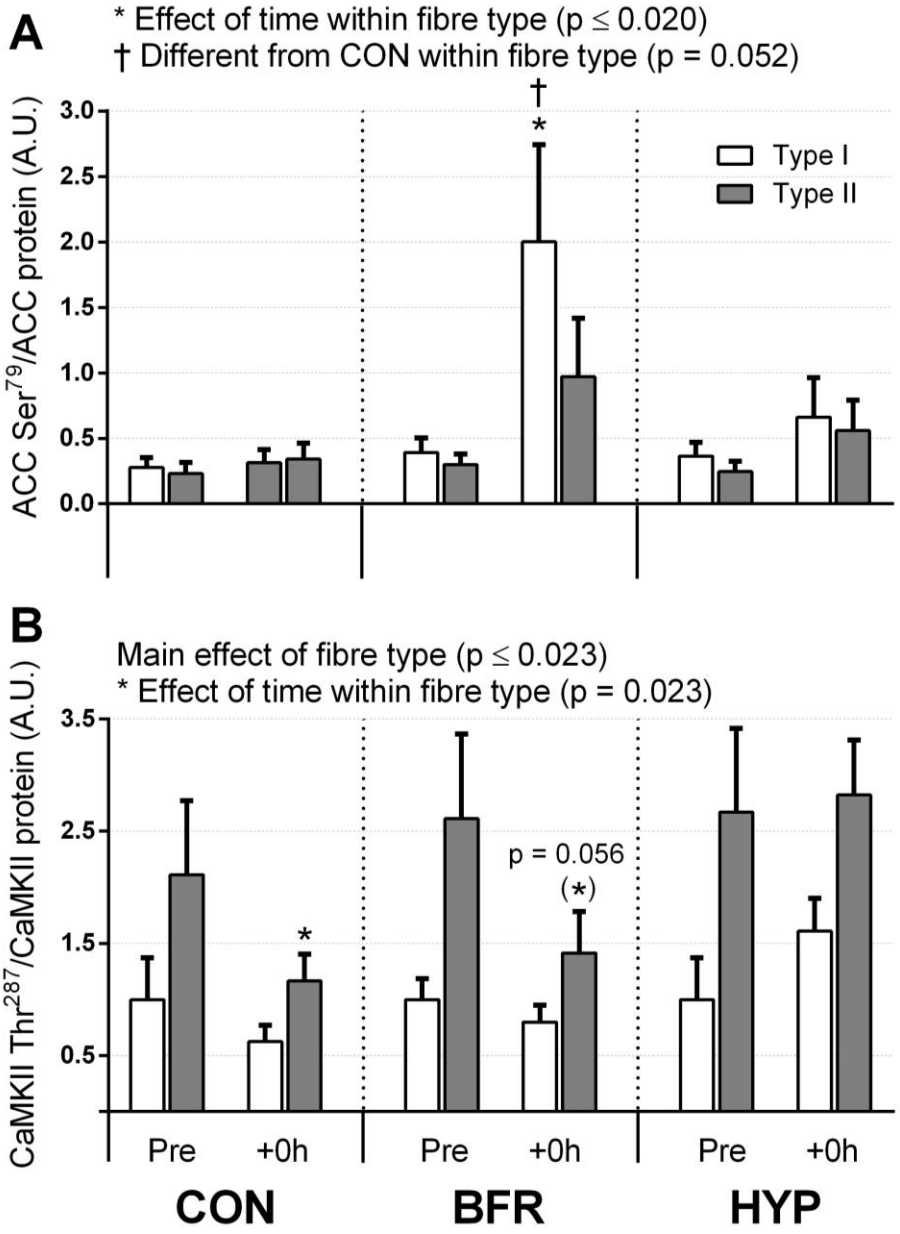

Figure 3.3. Phosphorylation of ACC at Ser[79] (A) and CaMKII at Thr[287] (B) in type I (slow-twitch) and II (fast-twitch) fibres from human skeletal muscle after an interval exercise session in normoxia (control, CON), systemic hypoxia (HYP), or with blood flow restriction (BFR).

3.1.5. Ca^{2+} signalling

Relative training intensity is considered an important variable for Na^+-K^+-ATPase-isoform adaptation (e.g. Fig. 1.2 in Chapter I). Since $[Ca^{2+}]_I$ increases in an intensity-dependent fashion and this has been linked with the degree of CaMKII activation (Aydin *et al.*, 2007), we examined whether Ca^{2+} signalling through activation of CaMKII, indicated by its phosphorylation at Thr[287], would be related to exercise-induced changes in expression of Na^+-

K^+-ATPase isoforms in human skeletal muscle (paper I). A novel finding was that CaMKII phosphorylation decreased in type II fibres after exercise sessions with and without BFR, but not after exercising in systemic hypoxia. In contrast, CaMKII phosphorylation was unchanged in type I fibres in all exercise conditions. If signalling via CaMKII was important for the mRNA responses of Na^+-K^+-ATPase isoforms, we would thus expect similar mRNA changes with BFR exercise and the exercise control, which would differ from changes with the session in systemic hypoxia. However, this was not the case. Specifically, exercise with BFR increased α_2 and FXYD1 mRNA, whereas exercise in systemic hypoxia elevated α_2 and β_1 mRNA. In contrast, the exercise control did not significantly change the levels of Na^+-K^+-ATPase mRNA transcripts. Because CaMKII phosphorylation at Thr^{287} reflects CaMKII autonomous activity in human muscle (Rose & Hargreaves, 2003; Rose *et al.*, 2006; Rose *et al.*, 2007), the present results support that modulation of CaMKII activity is not required for exercise-induced increases in levels of these mRNA transcripts in human skeletal muscle. CaMKII activity has been shown to be upregulated at the onset of exercise, from where it may gradually decrease towards resting level during sustained moderate-intensity exercise (Rose & Hargreaves, 2003). As such, the timing of the muscle biopsy after the end of the exercise session in paper I may not have been optimal for detecting changes in CaMKII phosphorylation (and activity) and is thus a limitation of the current experiment.

3.1.6. Association between alterations in Na^+,K^+-ATPase mRNA to a single exercise session and those of corresponding protein with exercise training

The present results of changes in Na^+,K^+-ATPase mRNA to different types of exercise with and without BFR and CWI are summarised in Table 3.1. These results will be discussed in the following section in relation to corresponding protein responses to several weeks of similar types of training, which are presented in Table 3.2. Fibre-type-specific protein results are specifically addressed later in this chapter. Because the effects of training on β_2 protein abundance could not be validly assessed (paper III), this isoform is excluded from the following discussion.

In the majority of the published studies on this topic, muscle mRNA and protein responses were not measured in the same individuals and different exercise protocols were used to compare these responses (cf. literature review in Chapter I). Thus, in paper III, we examined the responses of Na^+,K^+-ATPase mRNAs to a single session of intense interval cycling with those of corresponding proteins in response to performing the same type of training. After the first session, increases in α_1 and β_3 mRNA were observed (Table 3.1), whereas the training period caused an increase in α_1 and β_3 protein abundance (Table 3.2). Thus, increased protein turnover of these isoforms with intense training in human muscle appears, in part, to be a result of prior increases in the levels of constitutive mRNAs. This observation is consistent

with previous findings in mouse (Cougnon *et al.*, 2002) and rat muscles (Sharabani-Yosef *et al.*, 2002), where protein abundance of the α_1 isoform (no studies on β_3) follows the same pattern as that of the corresponding mRNA. In contrast, α_2 does not appear to be regulated at the mRNA level, supported by the selective increase in α_2 mRNA after post-exercise CWI, despite no change at the protein level with training plus CWI (paper III). This observation in human muscle is supported by a similar dissociation between responses of α_2 mRNA and protein to thyroid hormone in cultured skeletal muscle cells (Sharabani-Yosef *et al.*, 2002) and to sprint-interval training in rat EDL and soleus muscles (Rasmussen *et al.*, 2011). Together, these results suggest that the α_2 isoform may be predominantly regulated at the post-transcriptional level in mammalian skeletal muscles. Accordingly, in experiments with MDCK cells *in vitro*, exogenous overexpression of α-subunits repressed mRNA translation of the endogenous subunit (Clifford & Kaplan, 2009), indicating that translational repression is one mechanism by which the α-subunits are regulated at the post-transcriptional level.

As shown in Table 3.1, α_3 mRNA did not change with any exercise intervention studied for this thesis, although some of these interventions improved muscle K^+ regulation and/or exercise performance (paper II and III; Broatch *et al.*, 2017). Nor did the levels of α_3 protein change with sprint-interval training in paper III. Further, the α_3 isoform has been found to be lowly expressed at both the mRNA (Nordsborg *et al.*, 2005) and protein level (paper III) in human skeletal muscle. These results collectively suggest that the α_3 isoform is seldom altered by contractile activity and plays a minor role in regulating muscle contractile function in humans.

In paper III, β_1 mRNA increased after the first sprint-interval session (Table 3.1), whereas the sprint-interval training period raised β_1 protein in type II, but not in type I fibres (Table 3.2). In paper I, β_1 mRNA was unaltered by BFR exercise, and in paper II, BFR training did not affect β_1 protein in any fibre type. In contrast, the control leg training at moderate intensity (contralateral leg exercising without BFR) reduced β_1 protein abundance by 18% (paper II). The relatively low (<10%) measurement error for β_1 protein abundance reported in paper III (using the same conditions and antibody as in paper II), supports the likelihood of the latter result. This outcome may have been due to a detraining effect, given the low relative training intensity for the control leg and the reasonable training status of subjects (paper II). In contrast, a single session of the same type of exercise did not affect β_1 mRNA expression (paper I). Together, above results highlight that changes in β_1 protein abundance do not always correspond to changes at the mRNA level after a single training session. Because training caused fibre type-specific modulation of β_1 protein in paper III (Table 3.2), it can be speculated that β_1 mRNA may also be regulated at the fibre-type level to exercise in human skeletal muscle. The fibre-type-heterogeneous nature of our samples for mRNA analysis may thus have been a limitation of paper I.

As highlighted in the above section, a single session of aerobic interval exercise with BFR increased muscle FXYD1 mRNA content (Table 3.1; paper I). Accordingly, six weeks of aerobic interval training with BFR resulted in elevated FXYD1 protein abundance in both muscle fibre types (Table 3.2; paper II). Collectively, these outcomes support that FXYD1 is partly regulated at the mRNA level by BFR training in human skeletal muscle. FXYD1 mRNA was neither affected by exercise in normoxia nor in systemic hypoxia (paper I). In agreement, FXYD1 abundance did not change after six weeks of aerobic interval training (control leg). Likewise, a single session of sprint-interval training did not affect FXYD1 mRNA, nor was FXYD1 protein abundance altered in type II fibres after six weeks of sprint-interval training. The latter findings expand observations in rat skeletal muscles of no effects of both endurance and sprint-interval exercise and training on expression of FXYD1 mRNA and protein, respectively (Rasmussen *et al.*, 2011). However, FXYD1 protein was reduced in type I fibres with the training period in paper III (finding specifically discussed later in this chapter; section 3.2.3), indicating that FXYD1 abundance may be regulated in a fibre type-specific fashion to certain types of training in human muscle. The use of whole-muscle samples for mRNA analysis, however, precluded us from examining fibre type-specific changes in FXYD1 transcript levels. Dissociation of mRNA and protein changes in this particular case may thus be ascribed to methodological limitations.

In summary, for the Na^+,K^+-ATPase isoforms α_1 and β_3, and FXYD1, increased protein abundance with training appears partly to be a consequence of elevated levels of constitutive mRNA. In contrast, our results support that the α_2 isoform may be regulated predominantly at the post-transcriptional level. For those isoforms that responded in a fibre type-dependent manner to training (β_1 in paper II and III, and FXYD1 in paper III), levels of corresponding mRNAs measured at the whole-muscle level were not altered. This could indicate that fibre type-heterogeneous samples are inappropriate to use to detect changes in these mRNA transcripts with exercise. This is underlined by the findings that the type of training that caused an increase in FXYD1 protein (paper II), also resulted in elevated FXYD1 mRNA determined at the whole-muscle level (paper I). Alternatively, the contribution from post-transcriptional regulation to increased protein turnover of these transcripts may vary with the type of exercise undertaken.

Table 3.1 Summary of findings from the current thesis related to effects of a single exercise session on mRNA content of Na^+,K^+-ATPase isoforms in human skeletal muscle

				Fold-changes in mRNA content of Na^+,K^+-ATPase isoforms						
Study	**Subjects**	**Exercise protocol**	**Metabolic changes**	**α_1**	**α_2**	**α_3**	**β_1**	**β_2**	**β_3**	**FXYD1**
Paper I	Recreationally active (trained) men (n=8) 57 mL/kg/min	Three sets of 3 x 2 min running bouts at 105% lactate threshold (~12 km/h), with bouts separated by 1 min and sets by 5 min. Blood flow restriction during bouts: ~175 mmHg.	Peak muscle lactate: ~50 mmol/kg d.w. muscle Peak blood lactate: ~6.7 mmol/L Minimum blood pH: ~7.2 Peak blood K^+: ~5.1 mmol/L	3 h: →	3 h: ~1.6↑	3 h: →	3 h: →	3 h: →	3 h: →	3 h: ~2.7↑
Paper I	Recreationally active (trained) men (n=8) 57 mL/kg/min	Three sets of 3 x 2 min running bouts at 105% lactate threshold (~12 km/h), with bouts separated by 1 min and sets by 5 min. Systemic hypoxia during bouts: ~3250 m altitude.	Peak muscle lactate: ~42 mmol/kg d.w. muscle Peak blood lactate: ~6.4 mmol/L Minimum blood pH: ~7.2 Peak blood K^+: ~5.1 mmol/L	3 h: →	3 h: ~1.6↑	3 h: →	3 h: ~1.7↑	3 h: →	3 h: →	3 h: →
Paper I	Recreationally active (trained) men (n=8) 57 mL/kg/min	Three sets of 3 x 2 min running bouts at 105% lactate threshold (~12 km/h), with bouts separated by 1 min and sets by 5 min.	Peak muscle lactate: ~28 mmol/kg d.w. muscle Peak blood lactate: ~3.5 mmol/L Minimum blood pH: ~7.25 Peak blood K^+: ~5.0 mmol/L	3 h: →	3 h: →	3 h: →	3 h: →	3 h: →	3 h: →	3 h: →
Paper III	Recreationally active men (n=10) 45 mL/kg/min	Four 30-s 'all-out' cycling sprints separated by 4 min of rest.	n/a	0 h: → 3 h: ~2↑	0 h: → 3 h: →	0 h: → 3 h: →	0 h: → 3 h: →	0 h: → 3 h: ↓ to 0.7	0 h: → 3 h: ~2↑	0 h: → 3 h: →
Paper III	Recreationally active men (n=9) 45 mL/kg/min	Four 30-s 'all-out' cycling sprints separated by 4 min of rest with post-exercise cold-water immersion (15 min at 10°C).	n/a	0 h: → 3 h: ~2↑	0 h: ~2.1↑ 3 h: →	0 h: → 3 h: →	0 h: → 3 h: →	0 h: → 3 h: ↓ to 0.7	0 h: → 3 h: ~2.5↑	0 h: → 3 h: →

↓ = decrease; ↑ increase; → no change

3.2. Training-induced adaptations in abundance of Na^+,K^+-ATPase isoforms in type I and II muscle fibres from humans

The following section provides a discussion of how the abundance of Na^+,K^+-ATPase isoforms is regulated in different muscle fibre types by exercise training in humans. Key findings from paper II and III related to this aim are summarised in Table 3.2, along with existing human findings on this topic, and will be discussed below.

3.2.1. Effects of training on α-isoform abundance in type I and II fibres

A novel finding from paper III was that six weeks of sprint-interval training increased (210 to 330%) α_1 abundance in both type I and II fibres in the skeletal muscle of recreationally-active men (Table 3.2). Thus, α_1 abundance is markedly upregulated in both muscle fibre types by sprint-interval training in humans. Given the primary role of α_1 is to sustain transmembrane Na^+/K^+ gradients in non-contracting fibres (Radzyukevich *et al.*, 2004; Radzyukevich *et al.*, 2013), this suggests that sprint-interval exercise requires a high capacity for maintaining basal Na^+/K^+ concentrations in both fibre types in recovery from exercise bouts within training. In agreement, four weeks of sprint-interval training resulted in an increased (29%) α_1 abundance in whole-muscle samples from similarly trained subjects (Iaia *et al.*, 2008). In contrast, aerobic interval training was without effect on α_1 abundance in type I and II fibres (paper II; Wyckelsma *et al.*, 2017). However, exercising with BFR resulted in a ~50% higher abundance of α_1 in both fibre types (paper II). Thus, α_1 abundance appears to be similarly regulated in type I and II skeletal muscle fibres by both aerobic and anaerobic interval training in humans, whereas training intensity may be an important factor in this regulation. The latter observation supports the positive association between exercise intensity and exercise-induced increases in α_1 mRNA expression in human muscle (Fig. 1.2, Chapter I). A beneficial effect of training intensity on α_1 abundance in both muscle fibre types is likely explained by marked perturbations in the metabolic environment in the fibres (Table 1.1, Chapter I), because such perturbations result in fluctuations in intracellular and extracellular ion (Na^+, K^+ and Ca^{2+}) concentrations (Nordsborg *et al.*, 2003b; Mohr *et al.*, 2004; Gunnarsson *et al.*, 2013) that have been linked to elevated α_1 expression (Hosoi *et al.*, 1997). Moreover, the time spent under severe metabolic stress, and thus the duration of intense exercise bouts, may also be important. For example, 4 to 8 weeks of sprint-interval training with a shorter sprint duration (4 to 6 seconds) compared to the above studies (30 seconds) had no effect on α_1 abundance in either type I or II fibres (Wyckelsma *et al.*, 2015) or in whole-muscle homogenates (Mohr *et al.*, 2007). Further, resistance training that is characterised by short maximal efforts interspersed by several minutes of rest was found to increase (79%) α_1 abundance in type II fibres (Perry *et al.*, 2016) and in whole-muscle samples (Dela *et al.*, 2004) in sedentary individuals. It may thus be

speculated that α_1 abundance is regulated at the fibre-type level according to the demand for α_1-stimulated ion transport (Radzyukevich *et al.*, 2004; Radzyukevich *et al.*, 2013), and thus periods of rest between maximal efforts could maximise α_1-stimulated ion transport and α_1 mRNA adaptation.

Exercise training comprising a high volume of exercise is an effective stimulus to increase α_2 abundance in the skeletal muscle of both untrained (Green *et al.*, 2004; Nielsen *et al.*, 2004; Benziane *et al.*, 2011) and recreationally active humans (Mohr *et al.*, 2007; Green *et al.*, 2008). Likewise, a large training volume appears to be required to elicit changes in muscle α_2 abundance at the fibre-type level. For example, an increase in α_2 abundance in type II fibres (30%) was observed after 576 minutes of aerobic interval training performed over twelve weeks (Wyckelsma *et al.*, 2017), whereas 324 minutes of similar training over six weeks was insufficient to alter α_2 abundance in type I and II fibres (paper II). Further, no significant change in α_2 abundance was detected in both fibre types after a period of sprint-interval training with a low exercise volume (45 min; paper III). In addition, whole-muscle α_2 abundance was elevated after six (Dela *et al.*, 2004), but not four weeks (Perry *et al.*, 2016) of resistance training in untrained subjects. However, a training-induced increase (76%) in α_2 abundance was evident in type I fibres in the latter study (Perry *et al.*, 2016), indicating six weeks of sprint-interval training is sufficient to invoke fibre type-specific changes in α_2 abundance. It was thus surprising that our sprint-training protocol in paper III (6 weeks) did not result in increases in α_2 abundance in any fibre type. However, a quantitatively higher α_2 abundance was observed in around three quarters of type I and II fibres (paper III). It is thus possible that the small sample size and great inter-subject variability prevented a statistically significant result (type-II error). Moreover, a non-significant increase (30%) in α_2 abundance was evident in type-II fibres after training with BFR (based on data from three subjects only, paper II), suggesting interval training with a high exercise duration and relative intensity may specifically increase α_2 abundance in type II fibres. This fibre type-specific tendency is in line with the concept that a greater proportion of type-II fibres must be recruited to sustain power output during continuous exercise (Kristensen *et al.*, 2015) when oxygen supply to skeletal muscle is compromised (paper II). Together, above findings indicate that training volume and (or) intensity must be high to increase α_2 abundance in different muscle fibre types in humans, whereas the demand for Na^+/K^+ transport invoked by training on each fibre type may also be important for the fibre-type dependent regulation of α_2 by training. This is supported by the observation that the major role of α_2 in skeletal muscle is to assure that contraction-stimulated increases in demand for Na^+/K^+ transport are met (Radzyukevich *et al.*, 2013).

In paper II and III, α_3 isoform abundance remained unchanged with the training period. This is consistent with observations in whole-muscle samples of unchanged α_3 abundance after three

weeks of aerobic interval cycling (8 x 5-min at 85% VO_{2max}) in well-trained men (Aughey *et al.*, 2007). Albeit only semi-quantitative, the western blots for α_3 in paper III suggest that this isoform is lowly expressed at the protein level in human skeletal muscle. This, along with the low α_3 mRNA expression detected previously in the same tissue (Aughey *et al.*, 2007), downplays the functional importance of α_3 for the muscle's contractile function in humans. This could be one explanation for the lack of change in α_3 abundance in both fibre types with the different types of training employed in the present thesis. In contrast to these observations, α_3 abundance was increased (31%) in whole-muscle samples after three consecutive days with 2 hours of cycling at 60% VO_{2max} per day (Green *et al.*, 2008), indicating exercise training can increase α_3 abundance in human muscle. The reason for the latter finding is unclear. Consensus from this discussion is that α_3 abundance is not altered in any muscle fibre type by most training regimens in humans and thus the role of this isoform for the muscle ion transport capacity may be insignificant.

3.2.2. Effects of training on β-isoform abundance in type I and II fibres

In a previous human study, four weeks of sprint-interval training selectively increased the abundance of β_1 in type II fibres (identified in individual fibre segments) (Wyckelsma *et al.*, 2015). In agreement, we found that β_1 abundance was higher (44%) after six weeks of sprint-interval training in type II fibres only (paper III). Given α-isoform abundance was increased in the same fibre type after training (paper III), this could indicate need for improved Na^+,K^+-ATPase activity in this fibre type with intense training in humans. In support, in rat gastrocnemius muscle, higher Na^+,K^+-ATPase hydrolytic activity was reported in membrane vesicles with a reduced (50 %) molar α_2/β_1 ratio caused by higher β_1 content, relative to vesicles with a greater ratio (1.0) (Lavoie *et al.*, 1997). In contrast to the selective increase in type-II fibre β_1 abundance by sprint-interval training, β_1 abundance decreased by 18% in type I fibres after six weeks of moderate-intensity aerobic interval training (paper II). The decrease may not have been a result of method variation, because technical variability in measurement was low (<12%). Given the training history and status of the subjects included in the latter study, this finding could rather indicate a detraining effect, as mentioned earlier. In agreement, adding BFR during exercise bouts abolished the decrease in type-I fibre β_1 abundance with the training period (paper II). Collectively, these results suggest that β_1 is regulated in a fibre type-specific manner to exercise training in human skeletal muscle. This regulation appears dependent on relative training intensity and thus the demands imposed by training on transmembrane Na^+/K^+ transport in the different fibre types.

The western blot data in paper III did not allow for quantification of possible effects of training on β_2 abundance. This was not due to issues with antibody validity as the β_2 antibody identified

the recombinant β_2 protein and a selective band at the expected molecular weight for positive control tissues (rat brain and EDL) and not for negative controls (paper III). It rather appears that β_2 is lowly expressed in human vastus lateralis muscle, because weak protein bands for β_2 were evident for all protein amounts of human muscle tissue loaded (8 to 32 µg), which corresponded to the signal intensity for rat soleus muscle (a negative control). A weak signal for β_2 was confirmed in single fibre samples before and after the training period (paper III). Whilst effects of training could not be assessed, the western blot results for β_2 in paper III clearly demonstrated a higher expression in type II compared to type I fibres. This observation is in agreement with a higher (27%) β_2 abundance in type II fibres previously identified in individual fibre segments (Wyckelsma *et al.*, 2015). As the K_m for Na^+ of α/β_2 heterodimers (7.5-13 mM) is higher than the corresponding K_m for α/β_1 complexes (4-5.5 mM) in rat skeletal muscles (Kristensen & Juel, 2010a), the current observation raises the possibility that human muscle Na^+,K^+-ATPase activity could be fibre type-dependent. However, even large changes in β_2 abundance in either fibre type with training may be of small relevance for ion transport function, because of the low expression of β_2 in human vastus lateralis muscle. Further research is warranted to determine if Na^+,K^+-ATPase activity is different between type I and II muscle fibres and whether it is altered in a fibre type-specific manner by exercise training in humans.

Na^+,K^+-ATPase β_3 abundance has been shown to be elevated in type I (1 fold) and II (3 fold) muscle fibres, and in whole-muscle homogenates (2.5 fold), with age in humans (McKenna *et al.*, 2012; Wyckelsma *et al.*, 2016). In rat skeletal muscles, a similar age-associated increase in β_3 abundance was reversed by fourteen weeks of endurance training (Ng *et al.*, 2003). Thus, regular continuous muscle activity potently attenuates age-induced increases in β_3 abundance in human muscle. In contrast, sprint-interval training increased β_3 abundance by more than 2 fold in both human muscle fibre types (paper III). Together, these findings demonstrate that β_3 abundance is similarly regulated in type I and II muscle fibres in humans and that this regulation appears to be dependent on the type of muscle activity (or lack thereof) regularly undertaken. In paper III, the increase in β_3 abundance occurred concomitant with an increase in α_1 abundance, suggesting an enhanced potential for α_1/β_3 complex assembly in both fibre types after the training period. This supports that the β_3 isoform could take part in maintenance of resting membrane potential in both fibre types, in line with the ion transport function of the α_1 isoform (Radzyukevich *et al.*, 2013). However, this warrants further investigation.

3.2.3. Effects of training on FXYD1 abundance in type I and II fibres

Previous human studies using whole-muscle samples reported no alterations in FXYD1 protein abundance following 10 days to 8 weeks of intense training (Thomassen *et al.*, 2010; Benziane *et al.*, 2011; Nordsborg *et al.*, 2012; Skovgaard *et al.*, 2014a). In contrast, in paper III, we found that six weeks of sprint-interval decreased FXYD1 abundance by 33 % in type I fibres. This decrease was reinforced by the large effect size (0.82), small confidence interval (22%), and reasonable reproducibility (coefficient of variation < 22%). FXYD1 abundance remained unchanged in type II fibres in the same study, highlighting for the first time that FXYD1 is regulated in a fibre type-dependent manner by intense training in human skeletal muscle. As discussed in Chapter I, FXYD1 may regulate Na^+,K^+-ATPase function in multiple ways. Thus, the present finding indicates that physiologically relevant adaptations could be overlooked by use of whole-muscle homogenate for protein analyses. Other methodological issues should also be avoided, including sample fractionation, i.e. removal of an indefinite amount of protein (Murphy & Lamb, 2013). In paper II, FXYD1 abundance was higher in both type I and II fibres after training with compared to without BFR, indicating relative exercise intensity is important for alterations in FXYD1 abundance at the fibre-type level with training in humans. The different regulation of FXYD1 abundance in type I fibres with training in paper II and III may be explained by differences in work-to-rest ratio, and/or training duration (approximately 45 vs. 324 minutes).

3.2.4. Summary

In summary, the abundance of α_1 and β_3 appears to be similarly altered in type I and II muscle fibres by different types of training in humans, whilst increases in α_1 abundance are likely training-intensity-dependent. Abundance of α_2 was not significantly increased in the studies included in this thesis, although a type-II error may have occurred probably due, in part, to a low sample size. While more research seems required, the existing studies suggest that this isoform may also be regulated by training according to intensity, but training volume could also be important. The α_3 isoform is likely lowly expressed in human skeletal muscle and its expression is hardly altered at the fibre type level by training in humans. In contrast, the β_1 is regulated at the fibre-type level by training in an intensity-dependent manner in human skeletal muscle. The β_2 isoform is likely lowly expressed in this tissue, such that changes in its abundance may be of little functional importance for the skeletal muscle ion transport capacity. Finally, FXYD1 is regulated at the fibre-type level by training in human skeletal muscle, which seems to be dependent on the type of training performed, as well as training duration.

Table 3.2 Fibre type-specific changes in Na^+,K^+-ATPase-isoform abundance with exercise training in human skeletal muscle

Study	Subjects	Training			Exercise performance	Measurement of K^+ handling	Whole-muscle Na^+,K^+-ATPase content	Na^+,K^+-ATPase-isoform abundance	
		Frequency and duration	***Protocol***	***Type***				***Type I fibres***	***Type II fibres***
Wyckelsma *et al.* (2015)	Recreationally active men (n=4) and women (n=5) 42 mL/kg/min	3 sessions/wk for 4 weeks	Three sets of 5 x 4-s all-out running sprints separated by 20 s of rest, with 4.5 min of rest between sets	Sprint-interval	n/a	n/a	n/a	α_1 α_2 α_3 ↔ β_1 β_2 β_3 ↔	β_1 ↑ 33% α_1 α_2 α_3 ↔ β_2 β_3 ↔
Perry *et al.* (2016)	Sedentary, healthy men (n=4) and women (n=2) 46 mL/kg/min	3 sessions/wk for 4 weeks	Three to four sets of 8-12 repetitions at 65-70% 1RM, with 1 min rest between sets (Leg-press, knee-extension, hamstring curl, and calf raise)	Resistance	Cycling time to exhaustion at 85% leg VO_{2max} ↑31%	Venous plasma $[K^+]$ ↔ $\Delta[K^+]$/work ratio ↔	[^{3}H]ouabain binding ↔ α_1 α_2 α_3 ↔ β_1 β_2 β_3 ↔	α_2 ↑ 76% α_3 ↑ 143% α_1 β_1 β_2 β_3 ↔	α_1 ↑ 79% β_1 ↑ 35% α_2 α_3 β_2 β_3 ↔
Wyckelsma *et al.* (2017)	Recreationally active older adults (69 years) men (n=6) and women (n=2)	3 sessions/wk for 12 weeks	4 x 4-min cycling bouts at 90-95% HR_{peak} separated by 4 min recovery at 50-60% HR_{peak}	Aerobic-interval	Aerobic peak power output during cycling ↑ 25%	Peak venous plasma $[K^+]$ ↑ (p=0.07) $\Delta[K^+]$/work ratio ↔	[^{3}H]ouabain binding ↑ 11% α_1 α_2 β_1 ↔	α_1 α_2 β_1 ↔	α_2 ↑ 30% α_1 β_1 ↔
Paper II	Recreationally active men (n=10) 50 mL/kg/min	3 sessions/wk for 6 weeks	Three series of 3 x 2-min cycling bouts at 60-80% peak aerobic power (1 and 2 min rest between bouts and series)	Aerobic-interval	Aerobic peak power output during cycling ↑ 11%	Arterial + Venous plasma $[K^+]$ ↓ a-v-K^+ difference ↔ thigh K^+ release ↔	n/a	β_1 ↓18% α_1 α_2 ↔ FXYD1 ↔	α_1 α_2 β_1 ↔ FXYD1 ↔
Paper II	Recreationally active men (n=10) 50 mL/kg/min	3 sessions/wk for 6 weeks	Three series of 3 x 2-min cycling bouts at 60-80% peak aerobic power (1 and 2 min rest between bouts and series) + blood flow restriction	Aerobic-interval + blood flow restriction	Aerobic peak power output during cycling ↑ 23%	Arterial + Venous plasma $[K^+]$ ↓ a-v-K^+ difference ↓ thigh K^+ release ↓	n/a	α_1 ↑ 46% (p=0.075) FXYD1 ↑ 108% α_2 β_1 ↔	α_1 ↑51% FXYD1 ↑ 60% α_2 β_1 ↔
Paper III	Recreationally active men (n=7) 45 mL/kg/min	3 sessions/wk for 6 weeks	4-6 x 30-s all-out cycling sprints separated by 4 min of rest	Sprint-interval	Aerobic peak power output during cycling ↑ 6% Incremental 2-km cycling time trial ↑ 3.3% 20-km cycling time trial ↔	n/a	n/a	α_1 ↑ 210% β_3 ↑ 300% FXYD1 ↓33% α_2 α_3 β_1 ↔	α_1 ↑ 270% β_1 ↑ 44% β_3 ↑ 410% α_2 α_3 FXYD1 ↔
Paper III	Recreationally active men (n=5) 45 mL/kg/min	3 sessions/wk for 6 weeks	4-6 x 30-s all-out cycling sprints separated by 4 min of rest + Cold-water immersion	Sprint-interval + Cold-water immersion	Aerobic peak power output during cycling ↑ 6% 2-km cycling time trial ↑ 2.4% 20-km cycling time trial ↔	n/a	n/a	α_1 ↑ 330% β_3 ↑ 540% FXYD1 ↓33% α_2 α_3 β_1 ↔	α_1 ↑ 260% β_1 ↑ 44% β_3 ↑ 400% α_2 α_3 ↔ FXYD1 ↔

3.3. Adaptations underlying improvements in skeletal muscle K^+ handling: Novel insights from blood flow-restricted training

In the published studies that have examined training-induced effects on Na^+,K^+-ATPase abundance in type I and II muscle fibres in humans, measurement of K^+ regulation was either not included (Wyckelsma *et al.*, 2015) or K^+ concentration was measured in venous plasma (Perry *et al.*, 2016; Wyckelsma *et al.*, 2017), which poorly reflects K^+ homeostasis at the muscle level (paper II; Nielsen *et al.*, 2004; Gunnarsson *et al.*, 2013). In the present thesis Na^+,K^+-ATPase-isoform abundance was assessed at the fibre-type level, together with measurement of net thigh K^+ release during isolated work with the quadriceps muscle (paper II). A novel observation was that higher abundance of β_1 (33%) and FXYD1 (108%) in type I, and α_1 (51%) and FXYD1 (60%) in type II fibres occurred concomitant with a reduced net rate of thigh K^+ release during intense exercise after training with BFR (Fig. 3.2). Furthermore, in the three subjects where α_2 abundance was determined, a large effect for an increase in α_2 was evident in both type I (38%, d = 0.8) and type II fibres (38%, d = 0.7) after the training period with BFR. In contrast, simultaneous training of the contralateral leg without BFR neither increased isoform levels nor altered thigh K^+ release. Collectively, these results suggest that adaptations in the expression of Na^+,K^+-ATPase isoforms at the fibre-type level are important for muscle K^+ regulation during exercise in humans. In support, an increased expression of these isoforms would inevitably improve the potential for assembly of more Na^+,K^+-ATPase complexes at the cell surface, thereby increasing the capacity for K^+ re-uptake (Clausen, 2003). Because microvascular permeability for K^+ is closely associated with blood perfusion (Friedman & DeRose, 1982; Kajimura *et al.*, 1998), the faster increase in blood flow at the onset of exercise in the BFR-trained leg may also have contributed to the reduced net rate of thigh K^+ release. However, because the rate of rise in K^+ concentration in both arterial and venous blood was lowered with BFR training (Supplemental Tables 1 and 2 in paper II), K^+ re-uptake by skeletal muscles, when compared to K^+ removal from the muscle interstitial space by the bloodstream, may have been the most influential adaptation underlying the reduced thigh K^+ release after training with BFR (paper II). Moreover, consistent with previous observations in humans (Nordsborg *et al.*, 2003b; Mohr *et al.*, 2004), enhancements in muscle K^+ regulation were temporally associated with an improved exercise tolerance after training with BFR (11% greater vs. control leg). This suggests that maintenance of muscle K^+ homeostasis is essential for performance during intense exercise in humans.

3.4. Development of a new method for fibre type identification of single muscle fibres

When first introduced to fibre-type-specific protein analysis in Assoc. Prof. Robyn Murphy's lab in 2015, I was surprised by the highly laborious and time-consuming nature of the analysis. I thought, are these laborious steps all necessary? Can we optimise this method? And can we do it differently? Regardless, I continued dissecting fibres and analysing Na^+,K^+-ATPase-isoform abundance in individual muscle fibres for paper III using modified western blotting (Murphy, 2011a). During this period, I visited Prof. Jørgen Wojtaszewski's lab to learn the dot blotting technique to be able to determine glycogen content in type I and II muscle fibres (Kristensen *et al.*, 2015). I was intrigued by the speed and simplicity of dot blotting. With this technique, glycogen content could be determined in single muscle fibres without the need for western blotting steps. I wondered if this dot blotting method could be used for fibre-type identification of single muscle fibres. When I returned to Australia, I immediately presented the idea to Robyn and we started testing it out. The first results were promising, so we continued with experiments. Ultimately, this work resulted in paper IV, and the main outcomes from the experiments will be discussed in the following section.

3.4.1 The dot blotting method for fibre type identification

In paper IV, a novel method predicated on dot blotting for fibre type identification of individual fibres from human muscle biopsies is described and validated. According to this method, individual fibre segments are collected from human muscle biopsies under a stereo microscope and separately placed in microfuge tubes containing a small volume (≤15 µL) of SDS buffer. After precipitation for an hour at room temperature to denature proteins, approximately one-tenth (1 to 1.5 µL) of each precipitated fibre segment is spotted with the use of a pipette onto two pieces of PVDF-membrane pre-activated in 96% ethanol. One of the membranes is then incubated with MHCI antibody and the other with MHCIIa antibody to examine the presence of MHC isoforms in each fibre segment. The membrane that was first incubated with MHCI is pre-incubated with a MHCIIx antibody. Differentiating between fibres expressing MHCI and MHCIIa on the same membrane is possible due to the different immuno-reactivity (IgG vs. IgM) of the antibodies used. In this way, fibres can be classified as type I, IIa, IIx, or hybrid (I/IIa or IIa/IIx). A membrane stripping step can be introduced between incubations with antibodies for enhanced visual identification of fibre type, as stains from previous incubations are removed by stripping prior to re-incubation with a second antibody. A stripping step is inappropriate for quantitative assessment of protein abundance (Murphy & Lamb, 2013), but in the context of (qualitative) fibre type identification, where a yes or no answer is sought, it may be appreciated by some researchers.

An example of fibre type identification using dot blotting is shown in Fig. 3.4 (next page). Twenty segments of individual fibres were isolated from a freeze-dried muscle sample. Each segment was dot blotted onto a PVDF-membrane as indicated by positions A1-10 and B1-10. Two samples of whole-muscle homogenate (control, + lane) was also spotted on the membrane. The result from probing with the MHCIIa antibody, revealed that 13 of the 20 fibres expressed the MHCIIa isoform (top blot in Fig. 3.4A). Probing the same membrane with the MHCI antibody indicated that two fibres expressed MHCI (red circles, middle blot). Re-probing the membrane with MHCIIx antibody revealed that four fibres, which were empty on the previous blots, stained positive (blue circles, lowest blot), and so these fibres were classified as type IIx. There was one remaining dot that remained negative for all three probes (green circles), indicating that the fibre segment may not have been placed in the microfuge tube, or the protein content of the segment may have been too low to be detected by dot blotting (e.g. due to a small amount of protein possibly being lost in membrane washing steps).

Fibre type identification using dot blotting was validated by western blotting. Results for the validation step is shown in Fig. 3.4B. For validation, one-fifth of each fibre from positions A1-10 and B1-10 was loaded onto two 4-15% stain-free gels, the first being sequentially probed with MHCIIa and MHCI antibody, and the second being probed with MHCIIx antibody. The 170 kDa indicator can be seen for each blot (lanes labelled M). A single stain-free gel is shown as a representation of the gels, and is indicative of the total protein loaded. The lanes are numbered according to the positions on the dot blots in (A) and below are indicated the assigned fibre type. Thirteen type IIa fibres (in black text), two type I fibres (red text), and four type IIx fibres (blue text) were positively identified. The fibre at position B10, which did not stain positive for any fibre type using dot blotting (Fig. 3.4A), was evidently a small fibre based on the intensity in the stain-free gel (Fig. 3.4B) and was thus characterised as a type IIx fibre using western blotting (green text). Based on these results, dot blotting is a valid method for fibre type assessment of individual fibres from human skeletal muscle biopsies.

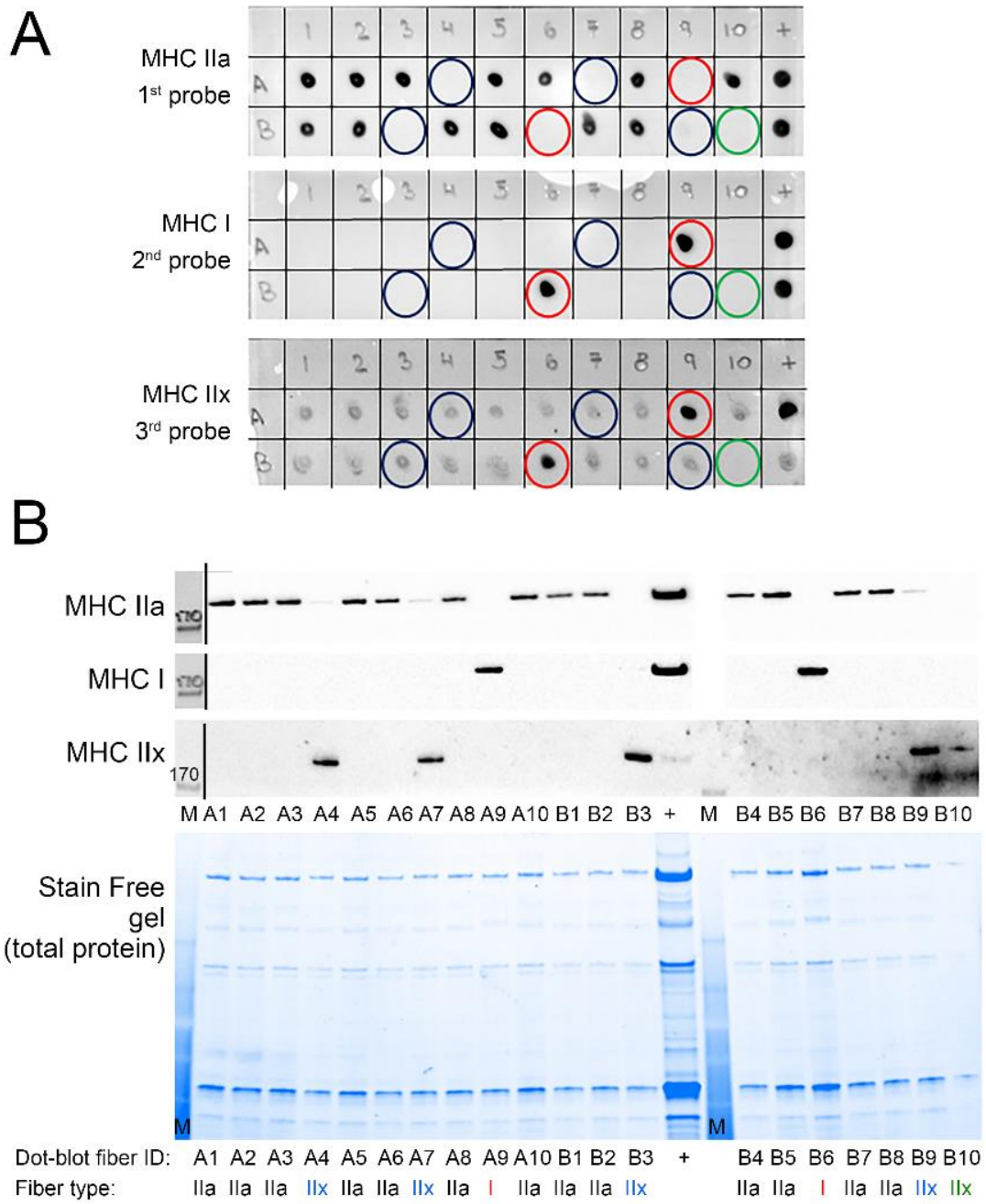

Figure 3.4. Validation of dot blotting for fibre type identification of single muscle fibres. Fibre segments classified as type I, IIa, IIx, and hybrid (I/IIa or IIa/IIx) according to presence of myosin myosin heavy chain isoforms using dot blotting (A) and western blotting (B).

The validity of dot blotting was further evaluated by quantifying abundance of proteins of varying size, known to be expressed in a fibre type-dependent manner in human muscle, in the same segments for which fibre type was identified on Fig. 3.4. Specifically, western blot membranes were probed for SERCA1, SERCA2a, CSQ1, CSQ2, Actin and AMPKβ2. As illustrated in Fig. 3.5, SERCA1 was only present in type II fibres, whereas SERCA2a was present only in type I fibres. Expression of calsequestrin (CSQ) was variable between fibre types, although CSQ1 (the fast type II isoform) was least abundant in type I fibres, which showed the highest expression of CSQ2 (the slow type I isoform). Actin (control) was similarly expressed in fibres of different types, which correspond to total protein in the stain-free gel. This was expected for a contractile protein that is abundant in both fast and slow-twitch muscle fibres. Also as expected, AMPKβ2 showed the most variability in expression across fibre types, regardless of MHC isoforms present.

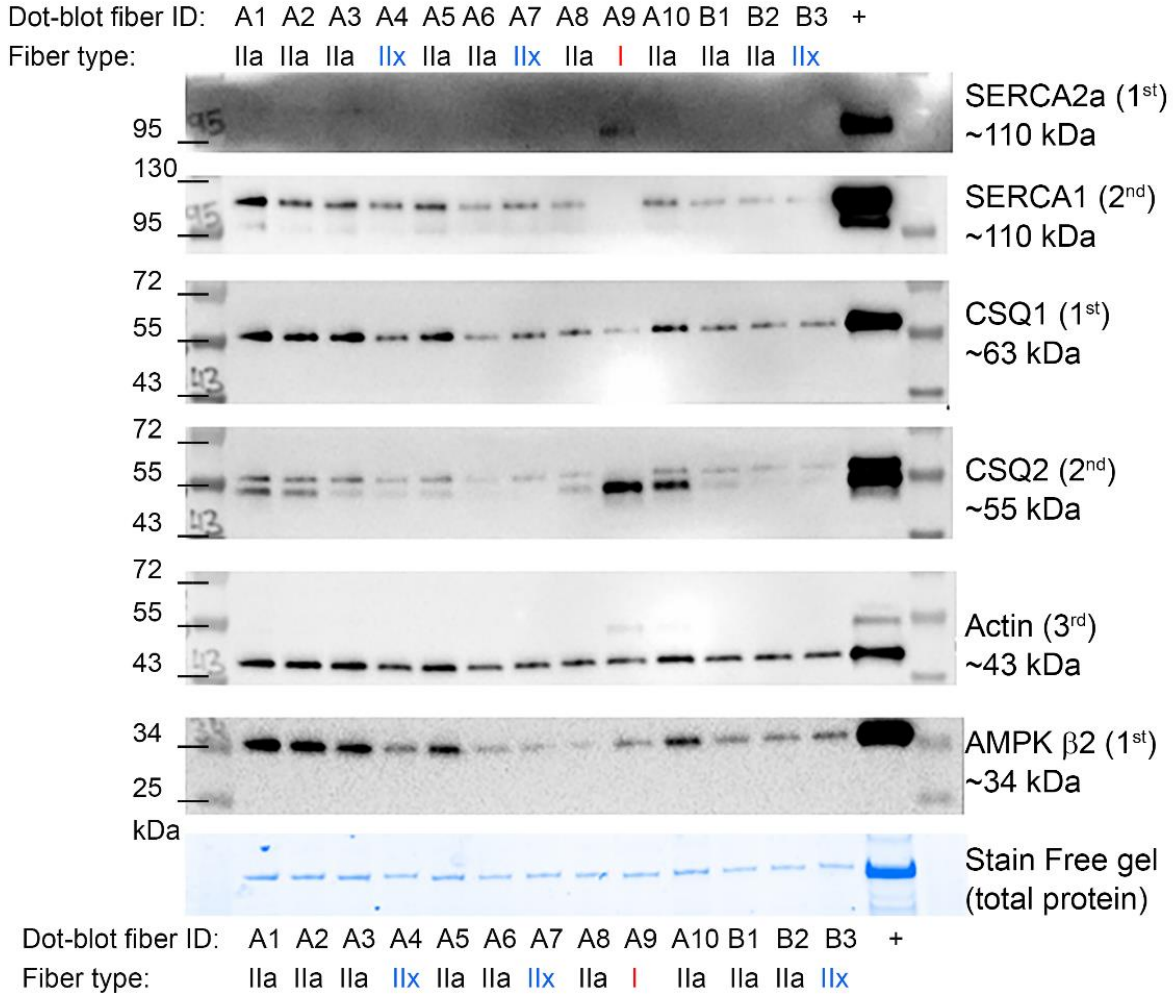

Figure 3.5. Fibre type-specific expression of proteins with different size in fibre segments classified as type I, IIa, or IIx according to dot blotting and western blotting in Fig. 3.4.

3.4.2 Summary

In summary, qualitative determination of fibre type of an isolated muscle fibre can be obtained by use of dot blotting of one-fifth of a 1-3 mm fibre segment dissected from a human muscle biopsy. The work expands previous progress made in the ability to analyse proteins in segments of single skeletal muscle fibres (Murphy, 2011b). This new method saves considerable time and money, because no gels or transfer step are needed and a less sensitive chemiluminescence reagent can be used compared to the latest advance in methodology predicated on western blotting (Murphy, 2011a). Previously, protein densitometry analyses had to be performed separately for each fibre segment before averaging the data, which markedly increased the time required for fibre-specific analysis. With dot blotting, rapid detection of fibre type precedes pooling of fibres, and proteins can then be quantified at the pooled-fibre level using modified western blotting (paper I, II, IV, Skovgaard *et al.*, 2018). Dot blotting for fibre type identification clearly opens possibilities for easy and rapid fibre type-specific analyses, and thus for improvements in our understanding of how muscle fibre type may play a crucial regulatory role in skeletal muscle physiology.

3.5. Conclusions

The first part of the thesis explored the primary cellular mechanisms regulating the expression of Na^+,K^+-ATPase isoforms in human skeletal muscle. The novel insights gained from this part of the thesis were that increased oxidative stress, AMPK signalling, and perturbations in ion and redox homeostasis during training sessions are likely important determinants of training-induced increases in the skeletal muscle capacity for K^+ regulation and Na^+,K^+-ATPase-isoform expression in humans. In contrast, the level of hypoxia and lactate, and modulation of CaMKII signalling, in skeletal muscle may not play a significant role in training-induced regulation of Na^+,K^+-ATPase-isoform expression. Furthermore, increases in the skeletal muscle content of α_1, β_3, and FXYD1 with intense training could be partly mediated by elevated expression of constitutive mRNA. On the other hand, the α_2 isoform appears to be predominantly regulated at the post-transcriptional level. In addition, the present data indicate that using whole-muscle samples for mRNA analyses likely compromises the validity in assessment of the relationship between changes in mRNA and protein expression under circumstances, where protein content is regulated in a fibre type-dependent fashion (e.g. β_1 in paper II and III and FXYD1 in paper III).

The second part of the present thesis investigated the regulation of Na^+,K^+-ATPase-isoform abundance in type I and II muscle fibres by different types of exercise training in humans. The novel findings were that α_1 and β_3 isoforms are similarly regulated in type I and II muscle fibres by different types of training. In contrast, the β_1 isoform is regulated differently in different fibre types by training. The regulation of these isoforms (α_1, β_3, and β_1) appears to be training intensity-dependent. The α_2 and FXYD1 isoforms may also be regulated at the fibre-type level according to training intensity, but training volume could also be important for adaptations in levels of these isoforms. The α_3 and β_2 isoforms are lowly expressed in both type I and II muscle fibres from humans, whereas α_3 did not respond to the different types of training included in the thesis. These observations downplay the role of these isoforms for the muscle contractile function in humans. Further, the present results support that fibre type-dependent adaptations in expression of Na^+,K^+-ATPase isoforms are of significant relevance for the skeletal muscle capacity for K^+ regulation and exercise tolerance in humans. In addition, blood flow restriction appears to be a potent strategy to augment training-induced increases in performance and K^+ regulation during intense exercise in humans, whereas regular use of cold-water immersion after training sessions neither impairs nor improves adaptations in Na^+,K^+-ATPase-isoform abundance to intense training in humans.

The third part of the thesis evaluated the validity of dot blotting for fibre type identification of single muscle fibres from humans. The novel results from this part of the thesis are that dot blotting can be validly and reliably used for rapid and easy, qualitative determination of the fibre type of individual fibres from a human skeletal muscle sample. Furthermore, this new method saves considerable time and money, because no gels or transfer step are needed and a less sensitive (and cheaper) chemiluminescence reagent can be used. Dot blotting for fibre type identification clearly opens possibilities for improvements in our understanding of how muscle fibre type may play a crucial regulatory role in skeletal muscle physiology, as demonstrated in the current thesis.

3.6. Perspectives

As highlighted earlier in this chapter, there are a number of methodological limitations that precluded accurate assessment of the involvement of the primary mechanisms studied in the present thesis in regulating of Na^+,K^+-ATPase expression and the capacity for K^+ handling in humans. For example, phosphorylation status was used as surrogate marker of activity for AMPK and CaMKII. Further, measurement of ROS levels and oxidative damage *in vivo* is challenging and requires special equipment and personnel (Jackson, 2007; Jackson *et al.*, 2007). Oxidative damage was indirectly assessed in the thesis by measuring exercise-induced responses in levels of proteins and genes, known to be sensitive to changes in muscle oxidative damage (e.g. HSP27, HSP70, and catalase). More accurate and direct measurements of these molecular mechanisms are required to verify the current outcomes. Future studies could provide novel insights into the relationship between cellular mechanisms, mRNA and protein expression, and the skeletal muscle capacity for K^+ handling in humans by integrating measurements of Na^+,K^+-ATPase activity, interstitial K^+ level, or both. In fact, measurement of Na^+-stimulated Na^+,K^+-ATPase maximal *in vitro* activity (Juel *et al.*, 2013) was commenced using muscle samples from paper II. However, the analysis was unreliable in my hands (based on four repeated measurements in eight pre and post exercise samples) and thus it was discontinued and the results were excluded from the thesis. Furthermore, the demonstrated beneficial effects of blood-flow-restricted training on performance and K^+ regulation during intense exercise in humans open the possibility that this strategy could be useful for athletes, for which a great ability to perform high-intensity exercise is required. In agreement, the acute signalling response of skeletal muscle to exercise was augmented with BFR in our participants, despite their trained state (VO_{2max} = 57 mL/kg/min; paper I). In another study of trained men (VO_{2max} = 60 mL/kg/min), greater increases in the muscle's content of genes (eNOS and HIF-1α) that play roles in angiogenesis were observed after sprint-interval exercise when BFR was introduced in the recovery from the sprints (Taylor *et al.*, 2015). Clearly, the ergogenic potential of BFR training should be explored further in elite athletes,

who typically display a reduced responsiveness to exercise than their less-trained counterparts (Bloom *et al.*, 1976). In addition, the present thesis highlights that dot blotting can be validly used for rapid and easy fibre type identification of individual fibres from a human muscle sample. On this basis, it would be exciting to explore the potential of this new method for other fibre type-specific analyses, such as responses of genes, micro-mRNAs, and other proteins in response to various stimuli, such as exercise, nutrition, and disease.

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Paper I

Christiansen D, Murphy RM, Bangsbo J, Stathis CG, Bishop DJ (2018): Increased *FXYD1* and *PGC-1α* mRNA after blood flow-restricted running is related to fibre type-specific AMPK signalling and oxidative stress in human muscle. *Acta Physiol* (Oxf). 2018 Jan 31. doi: 10.1111/apha.13045. Impact factor = 5.93.

Received: 23 October 2017 | Revised: 1 January 2018 | Accepted: 24 January 2018

DOI: 10.1111/apha.13045

ORIGINAL ARTICLE

ACTA PHYSIOLOGICA

Increased *FXYD1* and *PGC-1α* mRNA after blood flow-restricted running is related to fibre type-specific AMPK signalling and oxidative stress in human muscle

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Funding information
International Postgraduate Research Scholarship granted to D. Christiansen

See Editorial Commentary: Ferguson, R. A. 2018. Blood flow-restricted exercise: Providing more bang for buck in trained athletes? *Acta Physiol* 223, e13065.

Abstract

Aim: This study explored the effects of blood flow restriction (BFR) on mRNA responses of PGC-1α (total, 1α1, and 1α4) and Na^+,K^+-ATPase isoforms (NKA; $\alpha_{1\text{-}3}$, $\beta_{1\text{-}3}$, and FXYD1) to an interval running session and determined whether these effects were related to increased oxidative stress, hypoxia, and fibre type-specific AMPK and CaMKII signalling, in human skeletal muscle.

Methods: In a randomized, crossover fashion, 8 healthy men (26 ± 5 year and 57.4 ± 6.3 mL kg^{-1} min^{-1}) completed 3 exercise sessions: without (CON) or with blood flow restriction (BFR), or in systemic hypoxia (HYP, ~3250 m). A muscle sample was collected before (Pre) and after exercise (+0 hour, +3 hours) to quantify mRNA, indicators of oxidative stress (HSP27 protein in type I and II fibres, and *catalase* and *HSP70* mRNA), metabolites, and α-AMPK Thr^{172}/α-AMPK, ACC Ser^{221}/ACC, CaMKII Thr^{287}/CaMKII, and PLBSer^{16}/PLB ratios in type I and II fibres.

Results: Muscle hypoxia (assessed by near-infrared spectroscopy) was matched between BFR and HYP, which was higher than CON (~90% vs ~70%; $P < .05$). The mRNA levels of FXYD1 and PGC-1α isoforms (*1α1* and *1α4*) increased in BFR only ($P < .05$) and were associated with increases in indicators of oxidative stress and type I fibre ACC Ser^{221}/ACC ratio, but dissociated from muscle hypoxia, lactate, and CaMKII signalling.

Conclusion: Blood flow restriction augmented exercise-induced increases in muscle *FXYD1* and *PGC-1α* mRNA in men. This effect was related to increased oxidative stress and fibre type-dependent AMPK signalling, but unrelated to the severity of muscle hypoxia, lactate accumulation, and modulation of fibre type-specific CaMKII signalling.

KEYWORDS
AMP-activated protein kinase, blood flow restriction, Na^+-K^+-ATPase, oxidative stress, PGC-1α, reactive oxygen species.

1 | INTRODUCTION

A decline in the ability to perform high-intensity exercise coincides with a critical threshold of locomotor muscle fatigue,[1] suggesting factors within or around skeletal muscle partly limit human exercise performance. One of these factors is the capacity for maintenance of resting transmembrane sodium (Na^+) and potassium (K^+) ion gradients,[2,3] which is determined primarily by the activity of the Na^+, K^+-ATPase (NKA).[4-6] The functional NKA complex is composed of several subunits, including a catalytic α, regulatory β, and an ancillary protein, phospholemman (FXYD), and these subunits are expressed as multiple isoforms ($\alpha_{1\text{-}3}$, $\beta_{1\text{-}3}$, and FXYD1) in human skeletal muscle.[7] Most of the isoforms have been shown to be regulated at the mRNA level by exercise,[8-13] and their relative distribution and assembly are critical for maximal NKA activity.[14] However, little is known about the effects of different ergogenic interventions (eg hypoxia and cold-water immersion) on exercise-induced modulation of the expression of these isoforms in human skeletal muscle.[7,11,15]

Another limiting factor for human exercise performance is the muscle capability to generate ATP via oxidative phosphorylation.[16] Accordingly, increases in both mitochondrial respiratory function and content (as assessed by citrate synthase activity) have been temporally related to enhanced exercise performance.[17] A key determinant of these endurance-type adaptations is the transcriptional co-activator, the peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α). It has recently been shown that human muscle contains different PGC-1α isoforms,[18] and these isoforms may regulate different aspects of the muscle response to exercise.[19] Furthermore, there is evidence for[18,20] and against[21,22] some of these isoforms (PGC-1α1 and PGC-1α4) responding to different types of exercise. But like the NKA isoforms, there is limited evidence about the effects of different ergogenic strategies on the regulation of the level of these isoforms in human muscle.

One potential strategy to augment increases in the expression of these isoforms could be to exercise with reduced muscle blood flow (blood flow restriction, BFR). To our knowledge, no study has explored the effects of BFR on exercise-induced mRNA responses of NKA isoforms and FXYD1 in skeletal muscle. Given these isoforms likely exert different functions in skeletal muscle[23,24] and are regulated at the mRNA level by muscle activity,[7,11] this would seem of great physiological relevance. Furthermore, although 3 studies have assessed the effects of BFR on exercise-induced changes in PGC-1α mRNA content in human muscle, only one study measured different PGC-1α transcripts.[25] In these studies, BFR either attenuated,[25] augmented,[26] or had no effect[27] on changes in PGC-1α mRNA content after exercise. These contradictory findings are likely related to the different experimental approaches used (eg type and intensity of exercise and the timing of BFR). Thus, to maximize the effectiveness of BFR training, there is need to improve our understanding of the physiological stressors involved in the regulation of the expression of NKA and PGC-1α isoforms and how these stressors can be influenced by BFR in humans.

Blood flow restriction has typically been achieved by inflation of an occlusion cuff fixed around the limb(s) proximal to locomotor muscles and has been applied during various exercise modes, including walking, cycling, and resistance training.[28-30] Inflation of the cuff compromises both the arterial and venous flow,[31,32] resulting in a hypoxic and more acidic intramuscular environment.[31] Successive deflation of the cuff promotes local reactive hyperaemia.[33] In combination, these mechanisms seem a powerful stimulus for amplifying the transient bursts in reactive oxygen species (ROS) levels and the resultant oxidative stress that accompany consecutive bouts of exercise.[34-36] In rat and human skeletal muscles, ROS appear required for exercise-induced increases in the mRNA content of the catalytic NKA isoforms (α_1, α_2, α_3),[10] and those of PGC-1α in skeletal muscle cells in vitro.[37] In the latter study, the effect of ROS was mediated by activation of the AMP-activated protein kinase (AMPK). Several human studies have reported simultaneous increases in muscle AMPK activation (as assessed by protein phosphorylation) and PGC-1α mRNA,[38-40] supporting AMPK may be involved in the regulation of these mRNA transcripts. However, it remains unknown in humans whether muscle oxidative stress may be related to the effect of BFR on NKA- and PGC-1α-isoform mRNA content in skeletal muscle, and whether this effect is associated with increased AMPK signalling.

Disturbances in blood flow during exercise invoked by BFR may also affect muscle ion (K^+ and Ca^{2+}) homeostasis by modulating the function of ion channels and transport systems, including NKA.[41,42] Furthermore, substantial increases in muscle release of, and interstitial and venous blood, lactate have been reported in response to BFR exercise.[31,43] Both modulation of intracellular ion concentrations[13,44] and lactate[45] have been implicated in transient, excitation-induced increases in NKA and/or PGC-1α mRNA levels. However, no study has explored if lactate is associated with exercise-induced increases in the mRNA content of NKA and PGC-1α isoforms in humans. In 2 independent cell culture studies, the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) was shown to mitigate increases in NKA and PGC-1α mRNA invoked by ionic perturbations in vitro.[13,46] Thus, activation of CaMKII due to ionic perturbations, or indirectly through stimulation of ROS production,[47] could be another mechanism by which BFR could augment increases in NKA and PGC-1α mRNA

content in human muscle. However, this hypothesis remains to be evaluated.

The first aim of this study was to explore the effect of BFR on changes in the mRNA content of PGC-1α (total and isoform 1α1 and 1α4) and NKA isoforms ($\alpha_{1\text{-}3}$, $\beta_{1\text{-}3}$, and FXYD1) in response to a single, moderate-intensity, interval exercise session in human skeletal muscle. The second aim was to elucidate some of the potential cellular stressors and molecular signalling proteins involved. Our working hypotheses were as follows: (1) BFR would augment the effect of exercise on the expression of NKA and PGC-1α isoforms, and (2) higher expression of these isoforms would coincide with increases in markers representative of responses to oxidative stress (HSP27 protein content in type I and II muscle fibres and whole-muscle *catalase* and *heat-shock protein 70*, *HSP70* mRNA), AMPK signalling (as assessed by the ACC Ser^{221}/ACC ratio), and CaMKII activation (as determined by CaMKII Thr^{287}/CaMKII). Evidence from astrocytes in vitro suggests the reperfusion phase and resulting tissue re-oxygenation, rather than hypoxia, may be a primary stimulus underlying increases in the expression of NKA isoforms in response to hypoxia-reperfusion.[48] Thus, we designed our BFR protocol to induce multiple bursts in hypoxia-reperfusion by incorporating repeated exercise bouts with BFR interspersed by periods with cuff deflation. We also included a hypoxic condition (ie exercising in normobaric, systemic hypoxia) to assess the hypothesis that (3) exercise-induced increases in isoform expression in the BFR condition would not be attributed to the concomitant muscle hypoxia.

2 | RESULTS

2.1 | Na^+,K^+-ATPase and FXYD1 mRNA transcripts (Figures 1 and 2)

NKAα_1 mRNA was not changed in BFR ($P = .90$, $d = 0.44$), in CON ($P = .39$, $d = 0.54$), or in HYP ($P = .43$, $d = 0.47$; Figure 1A). There were no significant differences among conditions for the change in *NKA*α_1 mRNA from Pre to +3 hours ($P \geq .54$, $d = 0.19\text{-}0.62$). *NKA*α_2 mRNA increased from Pre to +3 hours in BFR ($P = .050$, $d = 0.90$), but there was no change in CON ($P = .089$, $d = 1.1$) or in HYP ($P = .18$, $d = 1.0$; Figure 1B). There were no differences among conditions for the change in *NKA*α_2 mRNA from Pre to +3 hours ($P \geq .31$, $d = 0.26$ to 0.45). *NKA*α_3 mRNA was not changed in BFR ($P = .47$, $d = 0.57$), in CON ($P = .26$, $d = 0.63$), or in HYP ($P = .071$, $d = 1.1$; Figure 1C), and there were no differences among conditions for the change in *NKA*α_3 mRNA from Pre to +3 hours ($P \geq .31$, $d = 0.11\text{-}0.96$).

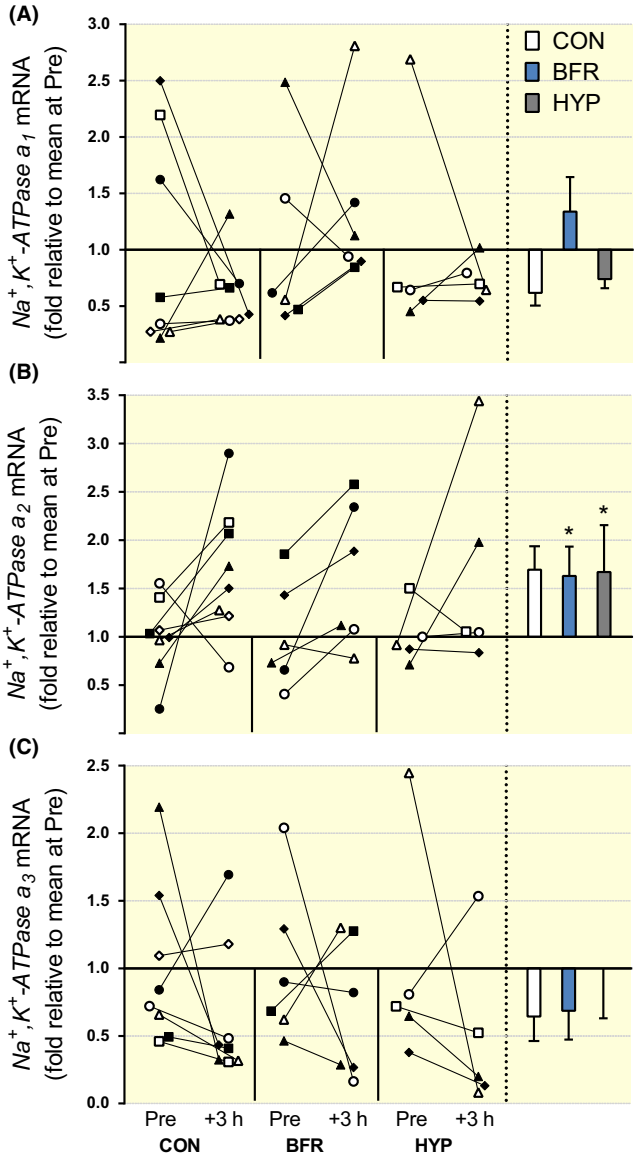

FIGURE 1 NKA-α-isoform mRNA responses to moderate-intensity interval running performed without or with blood flow restriction or in systemic hypoxia. (A) α_1, (B) α_2, and (C) α_3, mRNA content. Individual changes from before (Pre) to 3 hours after exercise (+3 hours) are displayed on the left with each symbol representing one participant across trials and figures. On the right are bars representing mean (±SEM) changes relative to Pre for exercise alone (CON, white; n = 8), with blood flow restriction (BFR, blue; n = 6) or in systemic hypoxia (HYP, grey; n = 5). *$P \leq .05$, different from Pre

NKAβ_1 mRNA increased from Pre to +3 hours in CON ($P = .049$, $d = 1.2$), but there was no change in BFR ($P = .064$, $d = 0.79$) or in HYP ($P = .077$, $d = 1.1$; Figure 2A). There were no differences among conditions for the change in *NKA*β_1 mRNA from Pre to +3 hours ($P \geq .47$, $d = 0.04\text{-}0.53$). *NKA*β_2 mRNA was not changed in BFR ($P = .69$, $d = 0.24$), in CON ($P = .51$, $d = 0.40$), or in HYP ($P = .55$, $d = 0.45$; Figure 2B), and there were

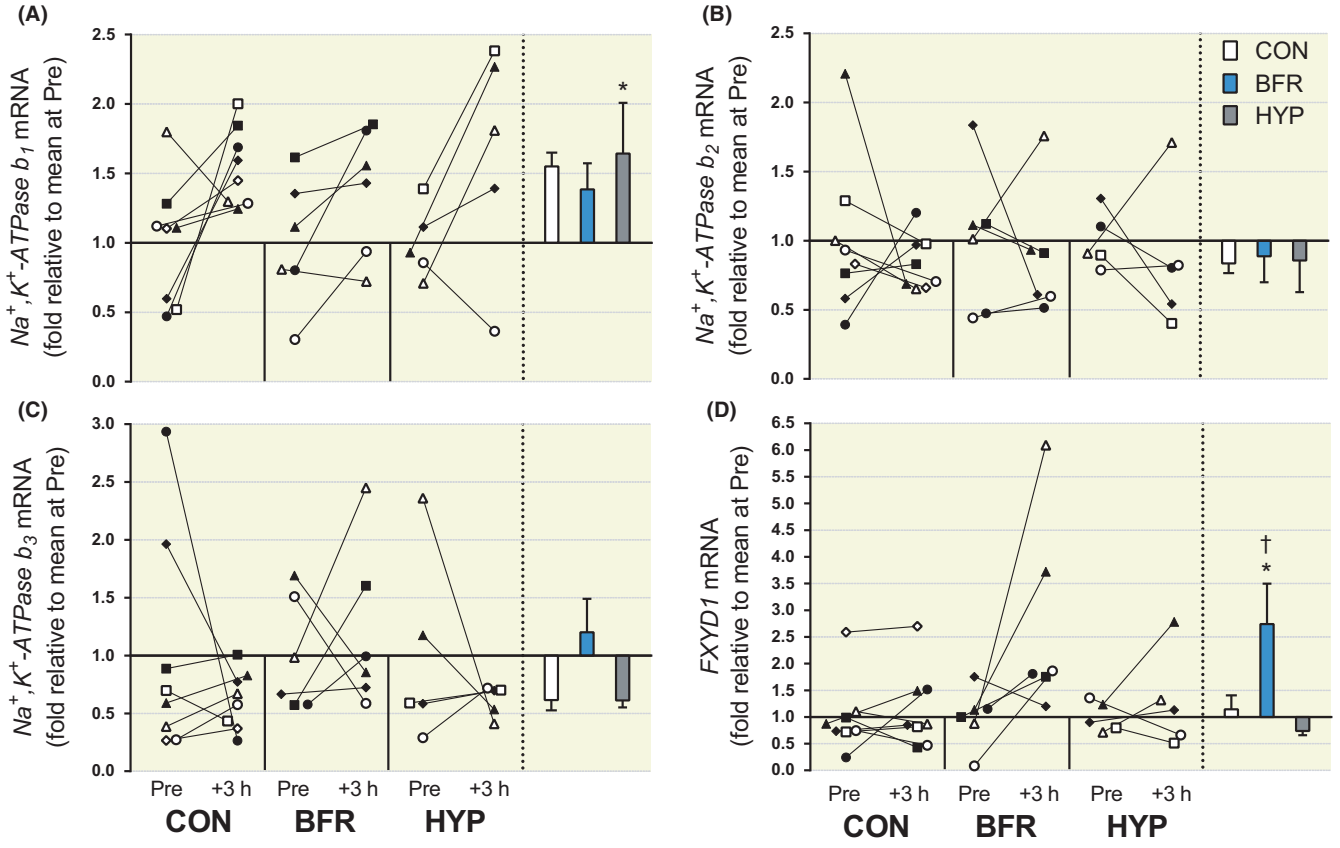

FIGURE 2 NKA-β-isoform and FXYD1 mRNA responses to moderate-intensity interval running performed without or with blood flow restriction or in systemic hypoxia. (A) β_1, (B) β_2, (C) β_3 and (D) FXYD1, mRNA content. Individual changes from before (Pre) to 3 hours after exercise (+3 hours) are displayed on the left with each symbol representing one participant across trials and figures. On the right are bars representing mean (±SEM) changes relative to Pre for exercise alone (CON, white; n = 8), with blood flow restriction (BFR, blue; n = 6) or in systemic hypoxia (HYP, grey; n = 5). *P < .05, different from Pre

no differences among conditions for the change in *NKA*β_2 mRNA from Pre to +3 hours ($P \geq .76$, d = 0.01-0.10). *NKA*β_3 mRNA was also not changed in BFR (P = .63, d = 0.34), in CON (P = .58, d = 0.55), or in HYP (P = .40, d = 0.74; Figure 2C), with no differences among conditions for the change in *NKA*β_3 mRNA from Pre to +3 hours ($P \geq .34$, d = 0.03-0.73).

FXYD1 mRNA increased from Pre to +3 hours in BFR (P = .058, d = 1.1), but there was no change in CON (P = .51, d = 0.20) or in HYP (P = .42, d = 51; Figure 2D). There were no differences among conditions for the change in *FXYD1* mRNA from Pre to +3 hours ($P \geq .19$), although the effect size was large (0.95) for the comparison BFR vs CON. The effect size was lower for the remaining comparisons: CON vs HYP (d = 0.33) and BFR vs HYP (d = 0.66).

2.2 | PGC-1α mRNA transcripts (Figure 3)

PGC-1α total mRNA increased from Pre to +3 hours in BFR (P = .031, d = 1.3) and in CON (P = .047, d = 1.1), but was not altered in HYP (P = .12, d = 1.1; Figure 3A). There were no differences among conditions for the change in *PGC-1α* total mRNA from Pre to +3 hours, although there was a low P-value (.088) and large effect size (1.11) for the comparison BFR vs CON. The P-value was higher and the effect size lower for the remaining comparisons: CON vs HYP (P = .36, d = 0.74) and BFR vs HYP (P = .38, d = 0.52). *PGC-1α1* mRNA increased from Pre to +3 hours in BFR (P = .010, d = 1.36), but was not altered in CON (P = .65, d = 0.29) or in HYP (P = .52, d = 0.35; Figure 3B). The increase in BFR was greater compared to that in CON (P = .047, d = 1.34), but not compared to that in HYP, although the P-value was low (.075) and the effect size large (1.19) for this comparison. There was no difference for the change from Pre to +3 hours between CON and HYP (P = .75, d = 21). *PGC-1α4* mRNA increased from Pre to +3 hours in BFR (P = .037, d = 1.3), but was not increased in CON (P = .10, d = 1.1) or in HYP (P = .18, d = 0.96). There were no differences among conditions for the change in *PGC-1α4* mRNA from Pre to +3 hours ($P \geq .11$), although the effect size was large (1.1) for the comparison BFR vs CON. The effect size was moderate for the remaining comparisons: CON vs HYP (0.58) and BFR vs HYP (0.61).

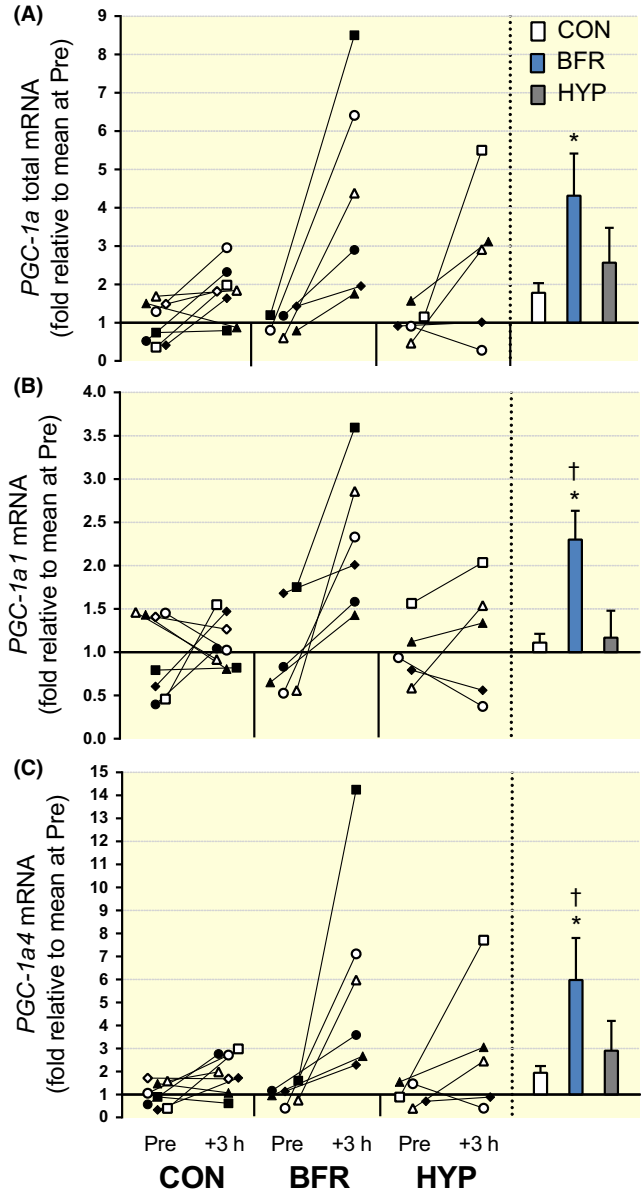

FIGURE 3 PGC-1α total and PGC-1α-isoform mRNA responses to moderate-intensity interval running performed without or with blood flow restriction or in systemic hypoxia. (A) *PGC-1α* total, (B) *PGC-1α1* and (C) *PGC-1α4*, mRNA content. Individual changes from before (Pre) to 3 hours after exercise (+3 hours) are displayed on the left with each symbol representing one participant across trials and figures. On the right are bars representing mean (±SEM) changes relative to Pre for exercise alone (CON, white; n = 8), with blood flow restriction (BFR, blue; n = 6) or in systemic hypoxia (HYP, grey; n = 5).*$P \leq .05$, different from Pre; †$P \leq .05$, different from CON and HYP

2.3 | Muscle hypoxia and indicators of responses to oxidative stress (Figure 4)

Muscle hypoxia, as assessed by deoxygenated haemoglobin concentration (muscle HHb), was higher during exercise in BFR ($P = .007$) and HYP ($P = .007$), relative to CON, except for the 7th bout of exercise ($P \geq .45$). During the recovery from the 1st ($P = .002$), 4th ($P = .005$), and 5th bout ($P = .016$), muscle HHb was higher in BFR, relative to CON. During the recovery from the 2nd bout, muscle HHb was higher in BFR ($P < .001$) and HYP ($P = .037$) compared to CON (Figure 4A). No differences were detected between BFR and HYP at any time point ($P = .1$). *Catalase* mRNA content increased in BFR ($P = .024$, $d = 0.70$), but there was no change in CON ($P = .881$, $d = 0.34$) or in HYP ($P = .505$, $d = 0.15$; Figure 4B). HSP27 protein content increased from Pre to +0 hour in both type I ($P = .003$) and type II fibres ($P = .004$) in BFR, with the increase in type II fibres being greater relative to CON ($P = .030$). No changes occurred in CON ($P \geq .80$) or in HYP ($P \geq .32$; Figure 4C). *HSP70* mRNA increased in BFR ($P = .057$, $d = 0.86$), but there was no significant change in CON ($P = .669$, $d = 0.18$) or in HYP ($P = .176$, $d = 0.95$; Figure 4D).

2.4 | Muscle metabolites (Figure 5)

ATP remained unchanged in all conditions ($P = .904$; Figure 5A). Lactate increased in BFR ($P < .001$) and in HYP ($P < .001$), but was not significantly changed in CON ($P = .075$). The increases in BFR and HYP were greater than CON ($P = .017$ and .015, respectively; Figure 5B). PCr decreased in CON ($P = .027$) and in HYP ($P = .011$), but was not altered in BFR ($P = .335$; Figure 5C). Similarly, Cr increased in CON ($P = .027$) and in HYP ($P = .011$), but was not changed in BFR ($P = .335$; Figure 5D). The PCr/Cr ratio decreased in CON ($P = .026$) and in HYP ($P = .018$), but was not altered in BFR ($P = .261$; Figure 5E). PCr and Cr content, and PCr/Cr ratio, were not different between conditions at Pre or +0 hour ($P > .05$).

2.5 | Venous blood lactate, pH, and K^+ concentration (Figure 6)

In BFR, blood lactate concentration ([lac^-]) increased ($P < .05$) after the 3rd exercise bout and remained elevated throughout the trial compared to rest. Blood [lac^-] was higher ($P < .05$) in BFR than in CON after the 3rd bout, the 5th to 9th bout, and after 3 minutes of recovery. In HYP, blood [lac^-] increased ($P < .05$) after the 3rd, 5th, 6th, 8th and 9th bout and in recovery, compared to rest. Blood [lac^-] was higher ($P < .05$) in HYP than in CON after the 3rd, 5th and 9th exercise bout. In CON, blood [lac^-] remained unchanged throughout the trial, compared to rest ($P > .05$; Figure 6A).

In BFR, blood pH dropped ($P < .05$) following the 1st exercise bout and remained lower ($P < .05$) compared to rest throughout the trial and 3 minutes into recovery, but returned to resting level after 6 minutes of recovery ($P > .05$). The drop in pH in BFR was lower, relative to

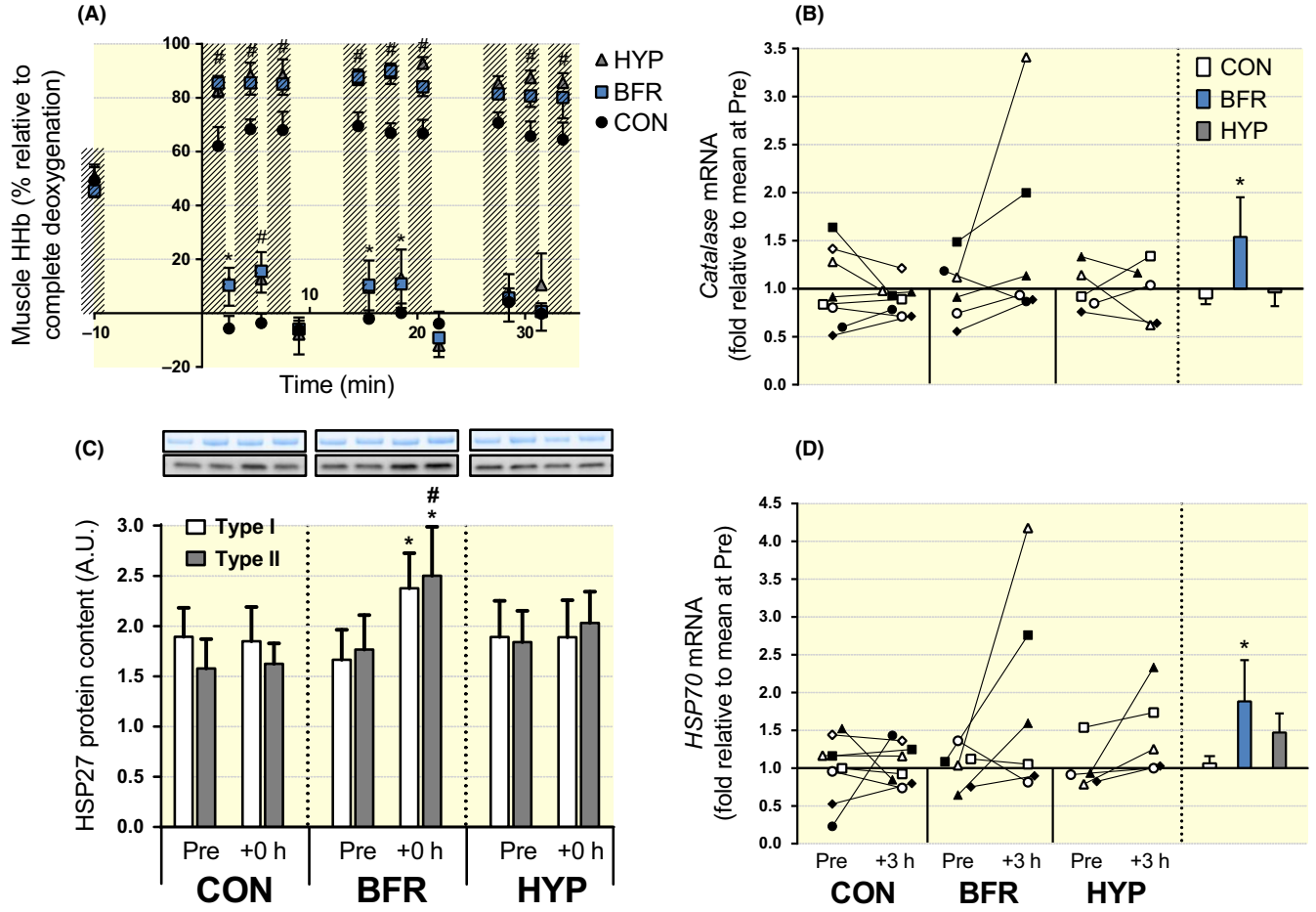

FIGURE 4 Changes in muscle hypoxia and indicators of responses to oxidative stress in response to moderate-intensity interval running performed without or with blood flow restriction or in systemic hypoxia. (A) Muscle hypoxia (ie deoxygenated haemoglobin, Muscle HHb) as assessed by near-infrared spectroscopy during moderate-intensity running without (CON, black symbols; n = 8) or with blood flow restriction (BFR, blue symbols; n = 8), or in systemic hypoxia (HYP, grey symbols; n = 8). Hashed bars represent exercise bouts. #$P \leq .05$, BFR and HYP different from CON. *$P \leq .05$, BFR different from CON. (B) Catalase, and (D) heat-shock protein 70 (HSP70), mRNA expression. Individual changes from before (Pre) to 3 hours after exercise (+3 hours) are displayed on the left with each symbol representing one participant across trials and figures. On the right are bars representing mean (±SEM) changes relative to Pre for moderate-intensity running without (CON, white) or with blood flow restriction (BFR, blue), or in systemic hypoxia (HYP, grey). *$P \leq .05$, different from Pre. (C) Heat-shock protein 27 protein content in type I (white bars) and type II fibres (grey bars) at rest before (Pre) and immediately after (+0 hour) exercise. Representative Western blots are indicated above the corresponding bars. *$P \leq .05$, increase vs Pre within BFR. #$P \leq .05$, BFR different from CON within fibre type. Data are means ± SEM

CON, after the 6th, before the 7th, and after the 8th and 9th, bout relative to CON ($P < .05$). In HYP, blood pH was lower, compared to rest, following the 3rd, 5th, and 6th bout, and before the 7th bout, but returned to resting level after the 7th bout, from where it remained unchanged. The drop in pH in HYP was lower following the 6th and before the 7th bout, relative to CON ($P < .05$). In CON, blood pH remained unchanged throughout the trial ($P > .05$; Figure 6B).

In all trials, blood K^+ concentration ($[K^+]$) increased after warm-up, and after the 1st to 8th exercise bout, compared to rest. In CON, blood $[K^+]$ was also elevated ($P < .05$) after the 9th bout, relative to rest. Compared to CON, blood $[K^+]$ was lower ($P < .05$) in BFR 4 minutes into recovery from the 3rd bout, and 6 minutes into recovery from the 9th bout, with no differences at other time points ($P > .05$), nor between HYP and CON at all time points ($P > .05$; Figure 6C).

2.6 | AMPK and ACC total and phosphorylated protein content (Figures 7 and 8)

Representative blots for AMPK and ACC are shown in Figure 7A.

In HYP, α-AMPK protein abundance decreased ($P = .023$) from Pre to +0 hour in type I, but did not change in type II ($P = .11$) fibres. In BFR and CON, α-AMPK abundance was not altered in either fibre type ($P \geq .42$; Figure 8A). The α-

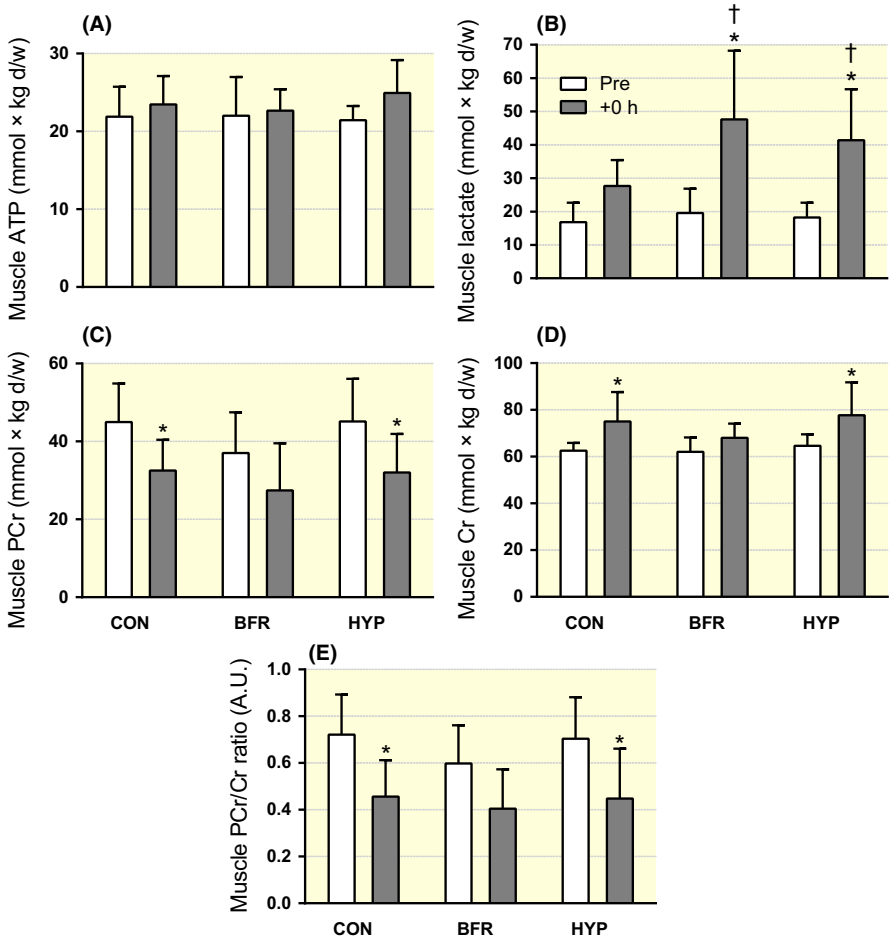

FIGURE 5 Changes in muscle ATP and lactate concentration in response to moderate-intensity interval running performed without (CON) or with blood flow restriction (BFR) or in systemic hypoxia (HYP). (A) ATP, (B) lactate, (C) phosphocreatine (PCr), (D) creatine (Cr) and (E) PCr/Cr ratio before (Pre, white) and immediately after exercise (+0 hour, grey). n = 8 for all conditions. Data are means ± SEM. *$P < .05$, different from Pre. †$P < .05$, different from CON

AMPK abundance was higher in type II vs type I fibres in all conditions ($P \leq .027$). In HYP, the phosphorylation of α-AMPK at Thr^{172} relative to total α-AMPK abundance (α-AMPK Thr^{172}/α-AMPK) increased in type I ($P = .003$), but not in type II ($P = .558$) fibres. In BFR and CON, there was no change in α-AMPK Thr^{172}/α-AMPK in either fibre type ($P \geq .11$). The α-AMPK Thr^{172}/α-AMPK was higher in type II vs type I fibres in all conditions ($P \leq .017$; Figure 8B).

ACC protein abundance was not altered in both fibre types in all conditions ($P \geq .17$), and overall, it was higher in type II vs type I fibres ($P \leq .015$; Figure 8C). The phosphorylation of ACC at Ser^{79} to total ACC abundance (ACC Ser^{79}/ACC) increased from Pre to +0 hour in type I fibres in BFR ($P \leq .020$), with the increase being higher relative to CON ($P = .052$). In the same condition, there was no change in ACC Ser^{79}/ACC in type II fibres ($P = .260$). No changes in ACC Ser^{79}/ACC occurred in CON and HYP ($P \geq .21$; Figure 8D).

2.7 | CaMKII and phospholamban total and phosphorylated protein content (Figures 7 and 9)

Representative blots for CaMKII and PLB are shown in Figure 7B.

CaMKII protein abundance did not change in either fibre type in all conditions ($P \geq .11$). In BFR and CON, it was higher in type I vs type II fibres (main effect of fibre type, $P \leq .038$), but not different between fibre types in HYP ($P = .20$; Figure 9A). Phosphorylation of CaMKII at Thr^{287} to total CaMKII abundance (CaMKII Thr^{287}/CaMKII) decreased in type II fibres in CON ($P = .023$) and tended to decrease in BFR ($P = .056$), but did not change in HYP ($P = .75$). No changes in CaMKII Thr^{287}/CaMKII occurred in type I fibres in any condition ($P \geq .21$). CaMKII Thr^{287}/CaMKII was significantly higher in type II vs type I fibres in all conditions (main effect of fibre type, $P \leq .023$; Figure 9B).

In type I fibres, PLB protein abundance decreased in CON ($P = .037$), whereas it did not change in BFR ($P = .79$) or in HYP ($P = .43$) in the same fibre type. PLB abundance did not change in type II fibres in any condition ($P \geq .29$). PLB abundance was lower in type II vs type I fibres (main effect of fibre type, $P \leq .050$; Figure 9C). The phosphorylation of PLB at Ser^{16} relative to total PLB abundance (PLB Ser^{16}/PLB) increased in type I fibres in CON ($P = .023$) and in BFR ($P = .010$), but it remained unchanged in HYP in the same fibre type ($P = .41$). In type II fibres, PLB Ser^{16}/PLB increased in BFR ($P \leq .026$) and in HYP ($P = .025$), but did not change in CON ($P = .35$; Figure 9D).

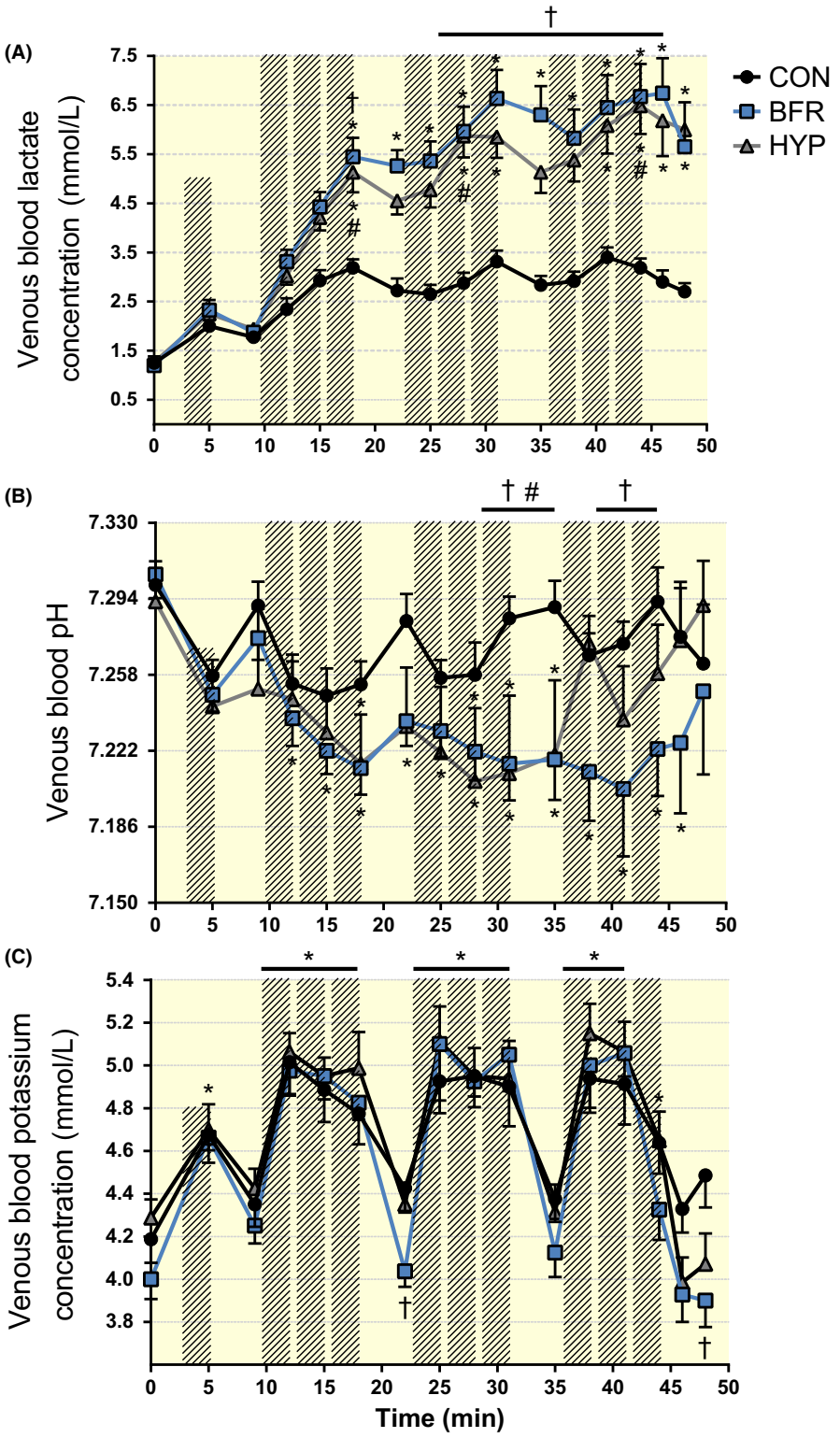

FIGURE 6 Changes in venous blood lactate, pH and potassium ion (K^+) concentration in response to moderate-intensity interval running performed without or with blood flow restriction or in systemic hypoxia. (A) Lactate, (B) pH and (C) K^+ concentration during moderate-intensity interval running without (CON, black symbols) or with blood flow restriction (BFR, blue symbols), or in systemic hypoxia (HYP, grey symbols). Hashed bars represent running bouts. n = 8 for all conditions. Data are means ± SEM. *$P < .05$, different from rest; †$P < .05$, BFR different from CON; #$P < .05$, HYP different from CON

3 | DISCUSSION

The main novel findings of the present study, which are summarized in Figure 10, were that moderate-intensity interval running performed with blood flow restriction (BFR) increased the mRNA content of the NKA regulatory subunit, FXYD1 (~2.7-fold), and of PGC-1α (total, 4.3-fold), 1α1 (2.3-fold), and 1α4 (6-fold), in human skeletal muscle. These responses to BFR were associated with increases in indicators of responses to oxidative stress (HSP27 protein in both fibre types, 70%; *catalase* and *HSP70* mRNA, 1.5- to 1.9-fold) and fibre type-dependent AMPK downstream signalling, reflected by elevated (2-fold) ACC Ser^{79}/ACC ratio in type I, but not in type II,

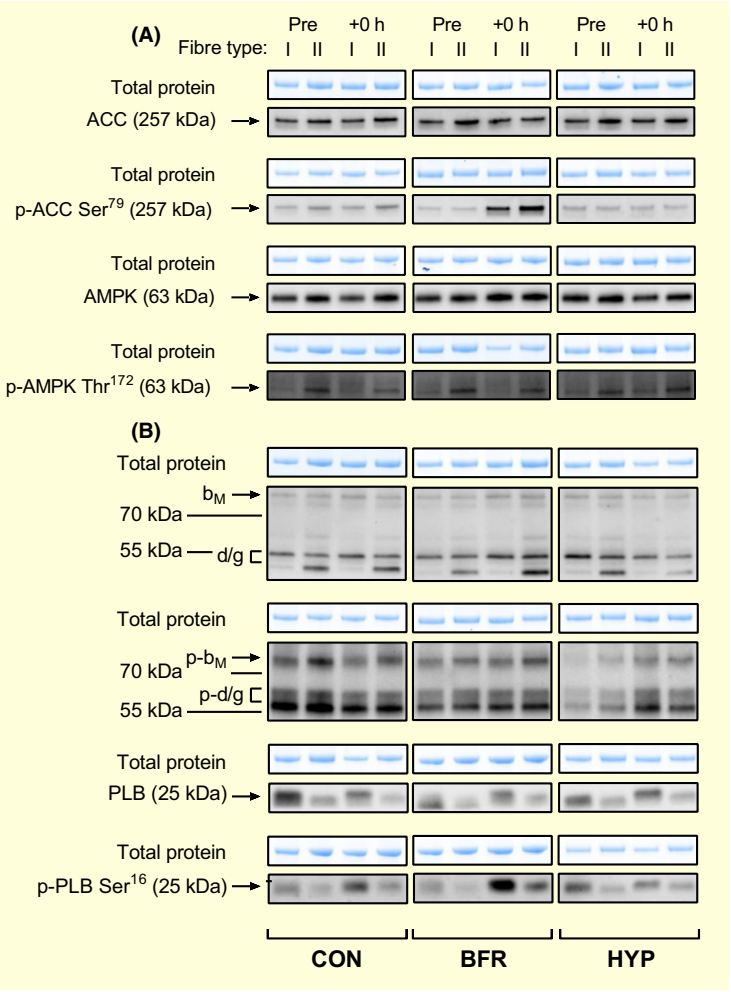

FIGURE 7 Representative blots for AMPKα, ACC, CaMKII and phospholamban (PLB) protein abundance and phosphorylation in type I and II human skeletal muscle fibres. Protein abundance and phosphorylation of (A) AMPK and ACC, and (B) CaMKII and PLB in human skeletal muscle in response to moderate-intensity interval running without (CON) or with blood flow restriction (BFR), or in systemic hypoxia (HYP) before (Pre) and immediately after (+0 hour) exercise. Total protein was determined in each lane from the stain-free gel images obtained after electrophoresis. CaMKII isoforms (β_M and σ/γ) are indicated in (B)

fibres. Furthermore, the effect of BFR on changes in FXYD1 and PGC-1α mRNA levels was unrelated to the severity of muscle hypoxia, lactate accumulation, and fibre type-specific modulation of the CaMKII Thr^{287}/CaMKII ratio.

3.1 | Blood flow restriction augments increases in FXYD1 mRNA content after moderate-intensity interval running in human skeletal muscle

A novel result was that *FXYD1* mRNA content increased (2.7-fold) due to BFR (Figure 2D). Despite similar increases in deoxygenated HHb between the BFR and HYP conditions (Figure 4A), systemic hypoxia was without impact on *FXYD1* expression. This suggests the magnitude of muscle hypoxia was not important for the BFR-induced increase in FXYD1. Nor may the increase be related to the severity of metabolic stress, as muscle lactate increased, whereas PCr content and PCr/Cr ratio decreased, to a similar level (+0 hour) in the BFR and HYP condition (Figure 5). In contrast, the induction of *FXYD1* mRNA in BFR was accompanied by increases in indicators of responses to oxidative stress (Figure 4), implicating ROS in the regulation of FXYD1 mRNA by BFR exercise in human skeletal muscle. In agreement, FXYD1 overexpression has been shown to protect myocytes against ROS-induced NKA dysfunction,[49] highlighting a ROS-protective effect of elevated FXYD1 content. In cell culture, AMPK can be activated by ROS,[37] and this regulates *FXYD* transcription in mouse glycolytic skeletal muscles.[50] In line with these observations, we found that the increases in *FXYD1* mRNA and indicators of oxidative stress were paralleled by elevated AMPK downstream signalling, reflected by a higher ACC Ser^{79}/ACC ratio. Taken together, *FXYD1* mRNA content can be induced by a single session of BFR interval exercise in human skeletal muscle. This effect is likely related to greater oxidative stress and (or) AMPK activation. Moreover, the promoted *FXYD1* mRNA content in BFR was dissociated from changes in phosphorylated CaMKII and PLB in type I and II fibres (Figure 9), suggesting transcriptional upregulation of *FXYD1* mRNA content in human muscle does not require alterations in CaMKII autonomous activity.[51]

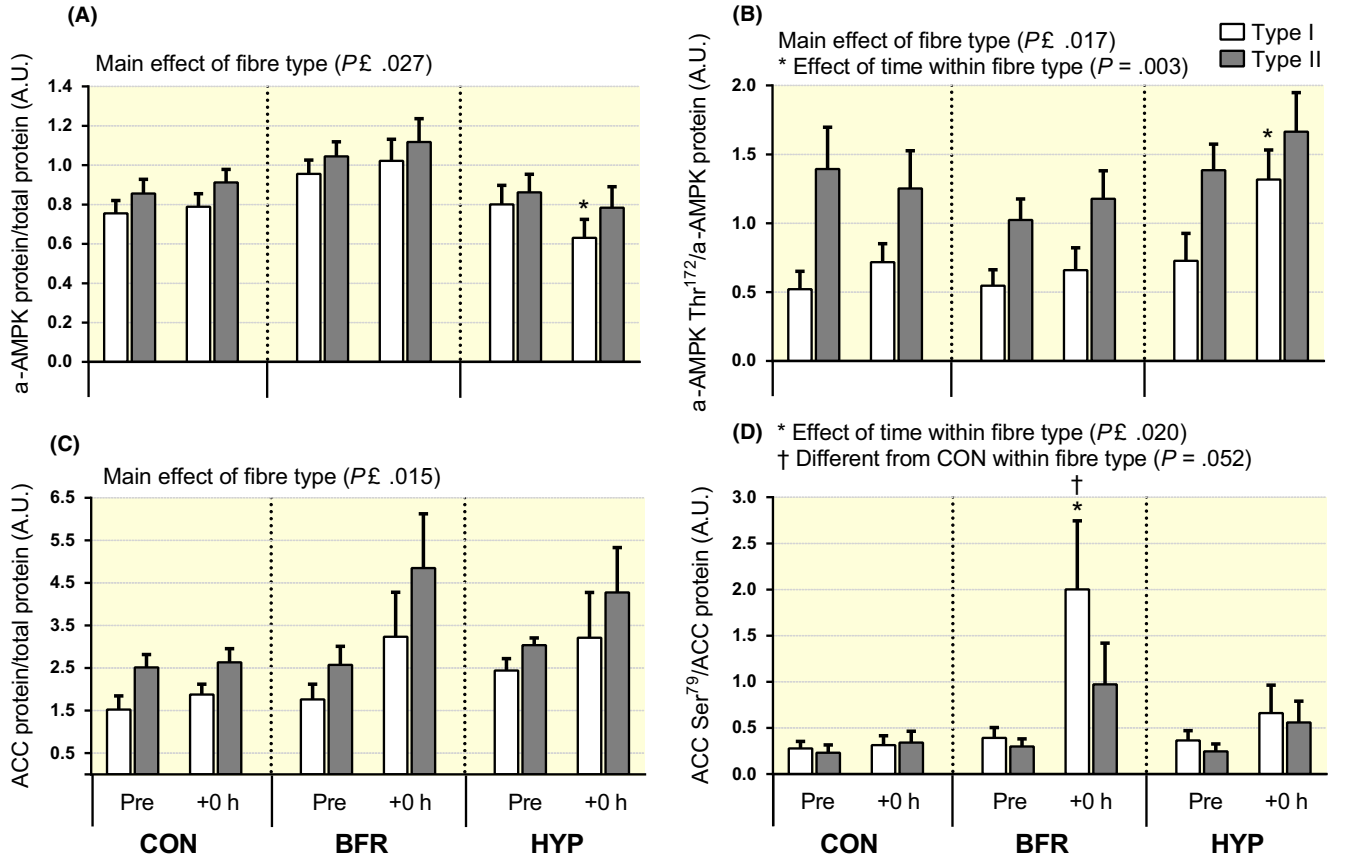

FIGURE 8 Changes in AMPKα and ACC protein abundance and phosphorylation in type I and II human skeletal muscle fibres in response to moderate-intensity interval running performed without or with blood flow restriction or in systemic hypoxia. (A) AMPKα protein, (B) AMPKα phosphorylation at Thr^{172} normalized to AMPKα protein, (C) ACC protein and (D) ACC phosphorylation at Ser^{79} normalized to ACC protein in type I (white bars) and type II (grey bars) fibres before (Pre) and immediately after (+0 hour) exercise. n = 8 for all conditions. Data are means ± SEM. *$P < .05$, different from rest within condition and fibre type; †$P \leq .05$, BFR different from HYP in (A), and from CON in (D)

3.2 | The effects of moderate-intensity interval running on Na^+,K^+-ATPase α_1 and β_3 mRNA content in human skeletal muscle are not influenced by blood flow restriction

The content of *NKA*α_1 and β_3 mRNA was unaffected by all exercise conditions (Figures 1A and 2C), despite pronounced differences among the conditions for changes in indicators of responses to oxidative stress, muscle hypoxia and lactate, and blood metabolites. This indicates the level of these mRNA transcripts are not severely affected by the nature of metabolic and ionic fluctuations, nor by the degree of hypoxia and oxidative stress, in human skeletal muscle. In support, raising the metabolic stress by performing simultaneous arm exercise was without effect on increases in muscle α_1 and β_3 mRNA content after isolated knee extensions.[52] Based on the individual changes for α_1 and β_3 in the present study (Figures 1A and 2C), these isoforms seem to be similarly regulated at the mRNA level in human muscle. For example, the same 2 individuals who decreased their α_1 mRNA content with BFR also reduced their β_3 expression in the same condition. Further, parallel and selective increases in α_1 and β_3 mRNA levels have been observed following sprint interval cycling with or without cold-water immersion in humans.[11] In another human study, *NKA*α_1 and β_3 were the only mRNA transcripts of those investigated ($\alpha_{1\text{-}3}$ and $\beta_{1\text{-}3}$) that remained unaltered in response to 45 minutes of cycling at 71% VO_{2max}.[10] Together, these results highlight α_1 and β_3 are likely regulated at the mRNA level by a similar pattern of cellular stress, which may be different from that (or those) important for changes in other NKA-isoform mRNA transcripts (eg compare the individual changes for α_1 and α_2; Figure 1A,B).

3.3 | The effects of blood flow restriction on changes in NKA α_2, β_1, β_2, and α_3 mRNA content in human skeletal muscle after moderate-intensity running

The NKA α_2 isoform is limiting for a muscle's contractile performance[53] and forms up to 90% of NKA complexes in

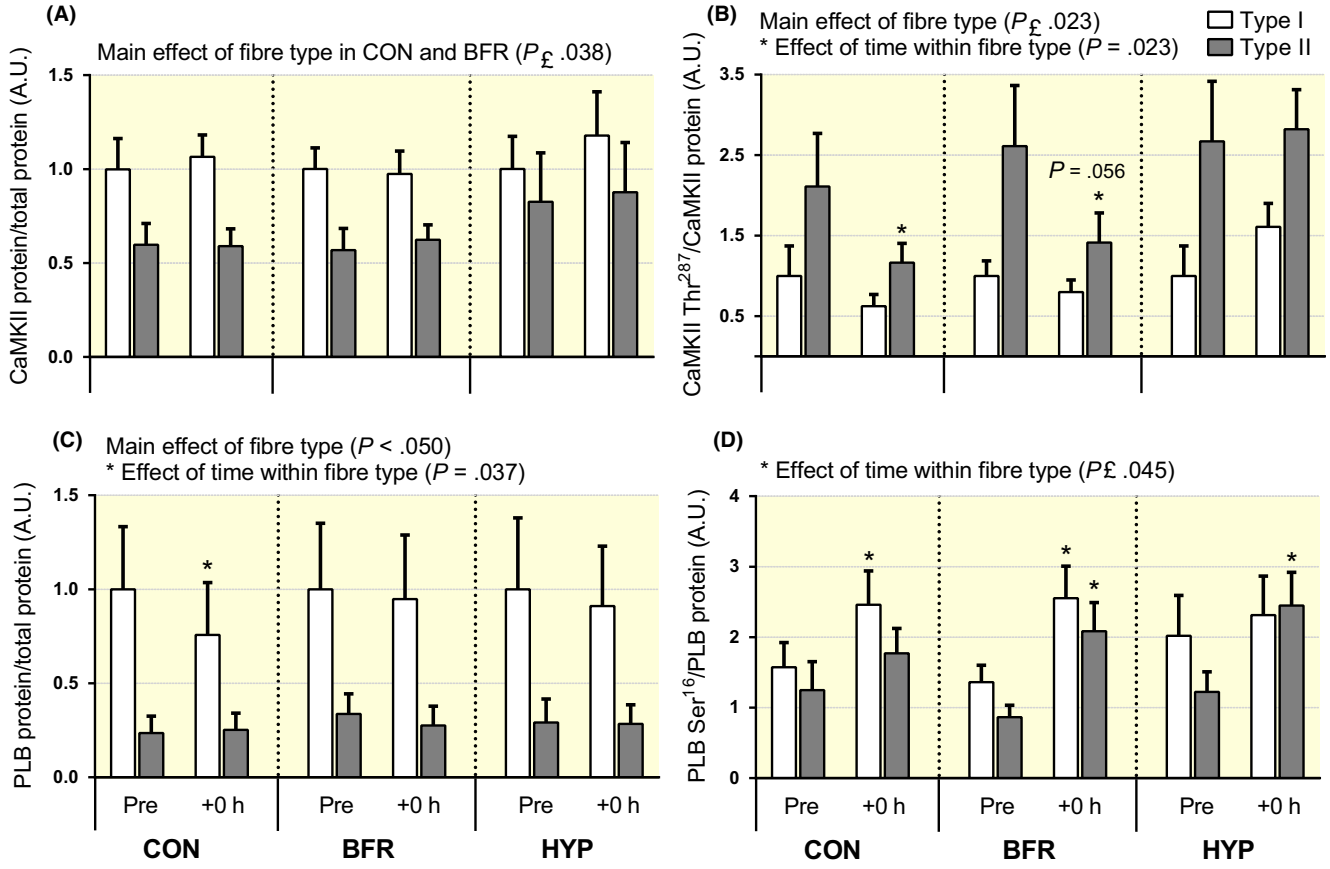

FIGURE 9 Changes in CaMKII and phospholamban (PLB) protein abundance and phosphorylation in type I and II human skeletal muscle fibres in response to moderate-intensity interval running performed without or with blood flow restriction or in systemic hypoxia. (A) CaMKII protein, (B) CaMKII phosphorylation at Thr^{287} normalized to CaMKII protein, (C) PLB protein and (D) PLB phosphorylation at Ser^{16} normalized to PLB protein in type I (white bars) and type II (grey bars) fibres before (Pre) and immediately after (+0 hour) exercise. n = 8 for all conditions. Data are means ± SEM. *P < .05, different from rest within condition and fibre type

adult rat skeletal muscles.[54] Together with the β_1 isoform, it constitutes the largest NKA pool in this tissue. Understanding the cellular stressors regulating α_2 and β_1 expression is therefore fundamental. In the present study, BFR significantly elevated *NKA*α_2 mRNA content (large effect), whereas it remained unaltered in CON and HYP. As the rise in the BFR condition was associated with increases in indicators of responses to oxidative stress, this could indicate ROS production may have been important for the potent effect of BFR on *NKA*α_2 mRNA in the present study. In accordance, ROS have previously been shown to play a role in the transcriptional induction of this isoform in human muscle.[10] However, the large effect size for CON and HYP (despite these conditions did not result in statistically significant gains) indicates the possibility of a statistical type II error for the change (Pre to +3 hours) in these conditions. Likewise, *NKA*β_1 mRNA was significantly elevated in CON (large effect), but not in BFR (P = .064) or in HYP (P = .077), despite a large effect (d = 0.8 and 1.1, respectively). These observations may have been due to the small sample size used and preclude us from unequivocally interpreting our data related to these isoforms. More research is required to clarify whether ROS production (or oxidative stress) is important for alterations in *NKA*α_2 mRNA in response to a session of BFR exercise in humans.

No changes in the mRNA levels of *NKA*α_3 and β_2 were found for any condition (Figures 1C and 2B). We have previously observed no change in human muscle α_3 mRNA content after sprint interval cycling of short duration (4-seconds sprints).[11] In contrast, exercise-induced increases in α_3 mRNA have been reported in other human studies. In these studies, induction of α_3 mRNA occurred immediately after exercise, with the level returning to basal state after 3 hours of recovery.[8,10,55] Thus, the time point of mRNA measurement may have influenced the current outcome and is a limitation of the present study. Another likely, at least contributing, explanation is the low expression of this transcript in human skeletal muscle[55] and so the noise is much higher (CV 3-fold higher vs other transcripts measured). The effect of a single session of exercise on β_2 mRNA content is controversial with human studies reporting either increased, decreased, or

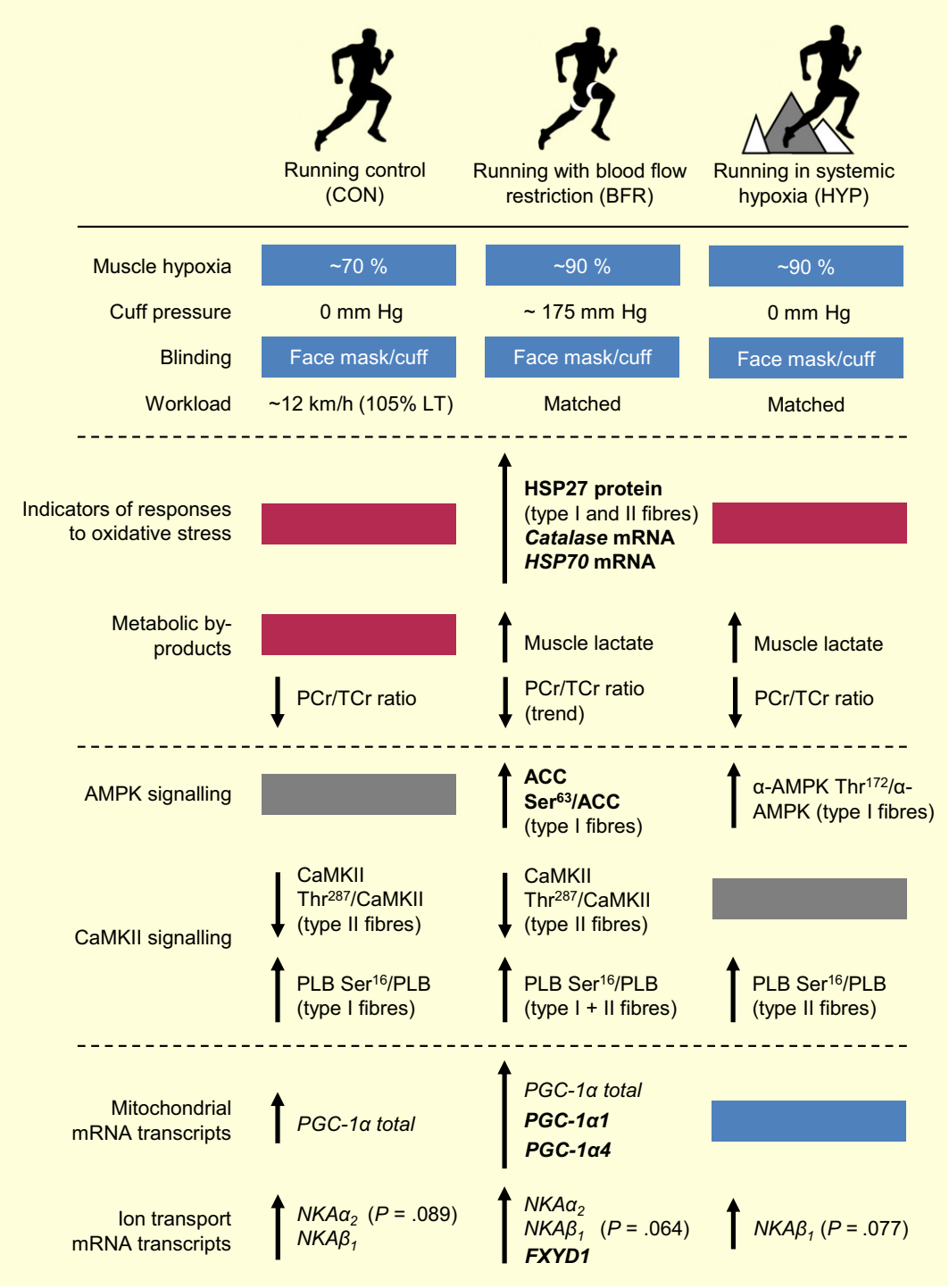

FIGURE 10 Summary of key findings. Effects of moderate-intensity interval running without (CON) or with blood flow restriction (BFR), or in normobaric, systemic hypoxia (HYP) on the mRNA content of Na^+,K^+-ATPase (*NKA*$\alpha_{1\text{-}3}$, *NKA*$\beta_{1\text{-}3}$, *FXYD1*) and PGC-1α (total, *1α1*, *1α4*) isoforms, indicators of responses to oxidative stress (HSP27 protein content in type I and II muscle fibres, *catalase* and *heat-shock protein 70*, *HSP70*, mRNA content), muscle hypoxia (ie deoxygenated haemoglobin as measured by near-infrared spectroscopy), lactate concentration, and AMPK and CaMKII signalling in the skeletal muscle of men. "p-" denotes phosphorylation; ACC, Acetyl-CoA carboxylase; AMPK, 5′ AMP-activated protein kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PLB, phospholamban; LT, lactate threshold

unchanged, levels 3 hours after the end of exercise. In the present study, the level of β_2 mRNA remained unchanged at the same time point. The reason for these conflicting findings is not clear,[11] and further mechanistic studies are necessary to understand how the β_2 isoform is regulated by exercise in human muscle.

3.4 | Blood flow restriction augments increases in PGC-1α total and isoform mRNA content after moderate-intensity running

A single session of moderate-intensity interval running raised the total muscle mRNA content of *PGC-1α* by 1.8-fold (Figure 3). This increase is small compared to those previously detected (8-fold to 10-fold) after endurance exercise sessions (eg 5 to 10 × 4 minutes at 90%-95% of VO_{2max}).[56-58] However, the smaller gain in the present study is not surprising given our use of a low relative exercise intensity (105% LT, ~12 km h^{-1}), the considerably high training status of our participants, and the positive relationship between exercise intensity and exercise-induced increases in muscle *PGC-1α* mRNA content previously reported.[39,59] Nevertheless, we chose this intensity as it was the highest tolerable mean speed, by which the exercise protocol could be performed with the chosen magnitude of BFR (cf. Materials and Methods). The small increase in *PGC-1α* mRNA could also relate to a low exercise volume, as our participants spent substantially less time exercising compared to the protocols previously studied (eg 5 to 10 × 4 minutes or 1 hour of cycling).[56-58]

In the present study, BFR augmented the exercise-induced increase in total *PGC-1α* mRNA (4.3-fold) and promoted the levels of the *1α1* (2.5-fold) and *1α4* (6-fold) transcripts (Figure 3). Consistent with these results, a reduction of ~15% to 20% in muscle blood flow during knee-extensor exercise (45 min at 26% of one-leg peak load) raised total *PGC-1α* mRNA in human skeletal muscle.[1,26] These findings contrast with 2 previous human studies that reported either attenuated[25] or unaltered[27] effects of BFR on exercise-induced change in skeletal muscle *PGC-1α* mRNA. The changes in *PGC-1α* levels in these studies may likely be explained by a low relative exercise intensity (40% of VO_{2max} in BFR vs 70% in CON) or the timing of BFR (15 s into passive recovery from each cycling sprint), respectively, as these conditions seem suboptimal for sufficient facilitation of the cellular stressors and signalling proteins involved in *PGC-1α* transcription.[39,59] In support, inductions of *PGC-1α* mRNA transcripts in the present study were closely related to the degree of oxidative stress and downstream AMPK signalling, which is discussed in detail below.

3.5 | Augmented PGC-1α-isoform mRNA content after blood flow-restricted exercise is related to muscle oxidative stress and AMPK signalling

A novel result was that the BFR-induced induction of *PGC-1α* mRNA levels was temporally associated with increases in indicators of responses to oxidative stress. Specifically, HSP27 protein increased in both fibre types, whereas *catalase* and *HSP70* mRNA was upregulated at the whole-muscle level. As these indicators are particularly sensitive to increases in hydrogen peroxide (H_2O_2) levels in either astrocytes[60] or myocytes,[61,62] these results indicate the effect of BFR on PGC-1α transcripts may have been mediated, in part, by exacerbated ROS production or the resultant oxidative stress. This is consistent with a previous observation in young men of an attenuated exercise-induced rise in *PGC-1α* mRNA after oral consumption of antioxidants (vitamin C and E),[63] and with those of a number of in vitro experiments where incubation of C_2C_{12} cells with H_2O_2 promoted *PGC-1α* mRNA content (1.4-fold) and promoter activity,[37] whereas pre-incubation with the ROS scavenger, N-acetylcysteine (NAC), abolished these effects.[37,64]

Treatment of C_2C_{12} cells with the AMPK activator, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), has been found to increase both *PGC-1α* mRNA (2.2-fold) and *PGC-1α* promoter activity (3.5-fold), and several AICAR-sensitive *PGC-1α* promoter sites have been identified.[65] In a more recent experiment, activation of *PGC-1α* transcription by ROS coincided with promoted AMPK activation.[37] In agreement with these in vitro observations, BFR provoked simultaneous exercise-induced increases in *PGC-1α*-isoform mRNA, indicators of responses to oxidative stress, and ACC Ser^{79}/ACC ratio (which strongly reflects AMPK activity[66]) in the present study. These findings support increased activation of AMPK could have been involved in the BFR-induced upregulation of *PGC-1α*-isoform mRNA levels. Factors other than ROS could partly account for the increased AMPK downstream signalling with BFR in the current study. For example, circulating noradrenaline can stimulate AMPK activity in skeletal muscle cells,[67] and BFR has been shown to exacerbate exercise-induced increases in circulating noradrenaline.[68] In addition, the increase in ACC Ser^{79}/ACC ratio was most pronounced in type I fibres (Figure 8D), indicating BFR-induced facilitation of AMPK signalling was fibre type-dependent. Consistent with this result, circulatory occlusion (250 mm Hg) accelerated glycogenolysis in type I fibres during repeated contractions in humans,[69] indicating altered metabolic activation of this fibre type during BFR exercise. Thus, future work should examine if modulation of exercise-induced mRNA responses by BFR is fibre type-specific. Moreover, inconsistent with the changes in ACC Ser^{79}/ACC ratio, α-AMPK Thr^{172}/α-AMPK was dissociated from increases in *PGC-1α* mRNA levels (eg differences between BFR and HYP). This result is in agreement with a number of previous human studies.[70,71] Dissociation of ACC Ser^{79}/ACC and α-AMPK Thr^{172}/α-AMPK in the present study could relate to different exercise effects on total abundance of these proteins (eg compare the mean values for α-AMPK protein after BFR vs HYP; Figure 8), and/or alternatively to the high sensitivity of ACC, in terms of phosphorylation, for (small) changes in AMPK activity.

3.6 | The effects of blood flow-restricted exercise on PGC-1α-isoform mRNA are unrelated to the severity of hypoxia, lactate accumulation, and modulation of fibre type-dependent CaMKII signalling in human skeletal muscle

In a previous human study, 1 hour of moderate-intensity cycling (60% of cycling peak power output) at simulated altitude (3000 m) had no effect on *PGC-1α* mRNA in the skeletal muscle of recreationally active men.[72] In agreement, *PGC-1α* total and isoform mRNA levels were unaffected by systemic hypoxia (3250 m) in the present study, despite deoxygenated HHb was matched between the BFR and HYP condition (Figure 4A). Thus, the severity of exercise-induced muscle hypoxia was not decisive for the effects of BFR on *PGC-1α* levels in the present study. In cardiac myocytes, long-term exposure to hypoxia (8 hours at 0.5% O_2) elevated *PGC-1α* mRNA by 3- to 6-fold,[73] indicating chronic exposure to hypoxia, at least in vitro, induces different mRNA responses compared to the intermittently hypoxic protocol applied in the present study. Given the comparable levels of muscle and blood metabolites after exercise with BFR or in systemic hypoxia (Figures 5 and 6), inadequate metabolic stress may also not account for the lack of an effect of systemic hypoxia. Rather, our results support the absence of an effect of systemic hypoxia was due, at least partly, to its insufficiency to substantially promote oxidative stress and activate AMPK compared to BFR. In addition, a novel result was that CaMKII Thr^{287}/CaMKII ratio decreased in type II fibres after exercise with (P = .056) and without BFR, but not when systemic hypoxia was superimposed. This is the first indicator in humans that decreased arterial oxygen saturation may affect contraction-stimulated CaMKII signalling in a fibre type-dependent manner in skeletal muscle. CaMKII phosphorylation at Thr^{287} has been positively correlated (r^2 = .884) with CaMKII autonomous activity in this tissue.[51] Given running in systemic hypoxia was without impact on *PGC-1α* levels, this indicates increases in the mRNA levels of PGC-1α isoforms in human muscle by a session of moderate-intensity running with BFR does not involve changes in CaMKII autonomous activity. It should be noted our data are limited to the time point immediately after exercise, whereas a transient increase (0.7- to 1.5-fold) in CaMKII autonomous, but not maximal activity has been detected early after the onset of moderate-intensity (76% VO_{2max}) cycling in human skeletal muscle.[74]

3.7 | Conclusion and perspectives

In summary, a single session consisting of moderate-intensity interval running with blood flow restriction augmented increases in the mRNA content of the NKA regulatory subunit, FXYD1, and of PGC-1α (total) and its isoforms 1α1 and 1α4, in the skeletal muscle of men. These effects of BFR were associated with increased oxidative stress and fibre type-specific AMPK downstream signalling, whereas the magnitude of muscle hypoxia, lactate accumulation, and fibre type-dependent modulation of CaMKII signalling was unlikely involved. Thus, intermittent BFR exercise is a potent strategy to augment the acute signalling and gene response associated with ion transport and mitochondrial adaptation in human skeletal muscle. Based on this work, future research should examine whether the effects of repeated interval exercise sessions with BFR over time could translate to improvements in the muscle capacity for K^+ handling and oxidative ATP production in humans.

4 | MATERIALS AND METHODS

4.1 | Ethical approval

This study was approved by the Human Research Ethics Committee of Victoria University, Melbourne, Australia (HRE14-309), and was performed in accordance with the latest instructions in the *Declaration of Helsinki*. Participants provided oral and written informed consent before enrolment in the study.

4.2 | Participants

Eight healthy men, engaged in team sports at a recreational level (5 in soccer, 2 in Australian-rules football and one in basketball), participated in the study. Their physical characteristics are shown in Table 1. All participants were non-smokers and engaged in their sport 2 to 4 times per week.

4.3 | Randomization and blinding

The study was a randomized, crossover experiment and took place in the Exercise Physiology Laboratory at the Institute of Sport, Exercise and Active Living (ISEAL), Victoria University, Melbourne, Australia. All sessions were performed on a Katana Sport XL treadmill (Lode, Groningen, Netherlands) in 21.4 ± 1.1°C and 40.8 ± 6.8% humidity. Participants completed 3 main trials matched for total work, duration (34 minutes) and work:rest ratio. These trials were separated by 1 week and consisted of interval running without (CON) or with blood flow restriction (BFR), or in normobaric, systemic hypoxia (HYP). Each participant was allocated a trial order using a random-number generator (MS Excel 2013, Microsoft, Redmond, WA, USA). To minimize any perceived placebo effect (not to be confused with a true placebo effect),[75] the participants were not informed about which trial was hypothesized to be of

greatest value to the physiological response, and whether they were breathing hypoxic or normoxic air. A pneumatic tourniquet (Riester, Jungingen, Germany) was attached to the participant's preferred kicking (dominant) leg by adhesive tape in all trials, but it was only inflated in BFR. In addition, the participants were informed that the study purpose was to evaluate the effect of different degrees of BFR. Information about what trial was to be performed on each occasion was given on the day of execution.

4.4 | Pre-testing

Prior to the main trials, the participants visited the laboratory on 4 separate occasions interspersed by at least 48 hours. On the first visit, participants performed a graded exercise test (GXT). This test was used to assess the participant's lactate threshold (LT) and maximum oxygen consumption (VO_{2max}). On the second visit, participants performed the BFR trial with near-infrared spectroscopy (NIRS) probes placed over the vastus lateralis muscle belly of their dominant leg to assess muscle oxygen content (cf. section on Muscle deoxygenation), and to accustom the participants to BFR and the equipment. During the third visit, participants completed the same running protocol with NIRS probes attached. The first 3 exercise bouts during this visit were performed without BFR or systemic hypoxia. The remaining 6 bouts were completed in normobaric, systemic hypoxia to accustom the participants to HYP and to allow estimation of individual inspired oxygen fraction (F_iO_2) to be used in HYP to match the level of muscle hypoxia during ISC (detailed in BFR and systemic hypoxia). The tourniquet was worn during both the second and third visit. The fourth visit consisted of a GXT similar to the one performed during the first visit. The LT from the fourth visit was used to determine individual running speed during the main trials (ie ISC, CON and HYP).

TABLE 1 Participant characteristics

Age (y)	26 ± 5
Height (cm)	177.3 ± 7.6
Body mass (kg)	74.3 ± 7.2
Body mass index (kg m^{-2})	23.6 ± 1.3
Upper thigh circumference (relaxed/contracted; cm)	57.5 ± 3.0/57.9 ± 2.8
Upper thigh skinfold thickness (mm)	8.2 ± 2.7
VO_{2max} (mL O_2 min^{-1})	4243 ± 408
VO_{2max} (mL O_2 kg^{-1} min^{-1})	57.4 ± 6.2
Peak treadmill speed during the GXT (km h^{-1})	14.9 ± 1.8
Lactate threshold (running speed in km h^{-1})	11.1 ± 1.6

GXT, graded exercise test.

Data are presented as mean ± SD. The lactate threshold was determined using the modified D_{max} method. Skinfold thickness was measured over the vastus lateralis muscle belly and is the mean of 3 consecutive measurements. Peak treadmill speed was calculated as the sum of the last completed stage and the product of the fractional time at the last stage and the increment (1 km h^{-1}).

4.5 | Main trials

On the days of the main trials, the participants reported to the laboratory between 8 and 9 AM after 7.3 ± 1.1 hours of sleep and after consuming a standardized dinner and breakfast (detailed in Diet and activity control) 15 and 2.5 hours, respectively, prior to arrival. After approx. 30 minutes of rest in the supine position, a catheter was inserted into an antecubital vein, allowing mixed-venous blood to be sampled. After an additional 15 minutes of rest, blood and muscle were sampled, also in the supine position. Next, the participants moved to the treadmill where they were instrumented with one pair of NIRS optodes on the belly of the vastus lateralis muscle of their preferred kicking (dominant) leg to reliably and non-invasively monitor muscle deoxygenation in vivo.[76] A belt was placed around their chest to measure heart rate. In a sitting position with the dominant leg unloaded, muscle deoxygenation was measured for at least 2 minutes until a plateau was reached and a stable baseline reading was recorded. Next, participants were fitted with a facemask covering the mouth and nose to enable them to breathe normoxic or hypoxic air. A pneumatic tourniquet was attached to the participant's dominant leg by adhesive tape. The tourniquet was inflated only in BFR before each bout of exercise and deflated upon termination of each bout. A time-aligned schematic representation of the experimental protocol is shown in Figure 11. Every trial commenced with a 5-minutes warm-up (WU) at 75% LT followed by 5 minutes of rest. At the third and fourth minute of the WU, a 5-seconds acceleration to ~110% LT, followed by a 5-seconds deceleration to 75% LT, was performed. Next, 3 series of three 2-minutes running bouts were executed at a fixed relative intensity (105% LT, 11.6 ± 1.7 km h^{-1}; no incline). The runs were separated by 1 minute, and the series by 5 minutes, of walking (~5 km h^{-1}), respectively. This design was introduced to promote repeated periods of hypoxia-reperfusion, which is a key stimulus for increases in NKA and PGC-1α mRNA in cell culture.[37,77] The duration of the running bouts and the work:rest ratio were based on pilot work and balanced to achieve the highest tolerable mean speed by which the exercise regimen could be completed with the chosen magnitude of BFR (3.0 mm Hg cm^{-1}). Antecubital venous blood was sampled at rest before exercise, prior to each series, immediately after each bout, and at 3 and 6 minutes after the end of exercise. Muscle was sampled at rest in the supine position before (Pre), immediately after (64 ± 28 seconds; +0 hour) and 3 hours post (+3 hours) exercise.

4.6 | BFR and systemic hypoxia

In all trials, a pneumatic tourniquet made of nylon with a width of 13 cm (Riester) was externally applied to the most proximal part of the participant's preferred kicking leg. In BFR, 15 seconds prior to the onset of a run, the tourniquet was rapidly inflated over ~10 seconds to reach an end-pressure of 3.0 mm Hg cm^{-1} (ie relative to thigh circumference, TCF; see below). The mean pressure was in the lower end of the range of pressures used in previous studies (~3-5 mm Hg cm^{-1}).[28,29,78-80] The pressure during running ranged from (mean ± SD) 123 ± 12 (range: 109-139) mm Hg in the float phase to 226 ± 24 (range: 200-260) mm Hg in the landing phase. The difference between our predetermined (~175 mm Hg) and actual (mean ± SD) pressure during the trials was −1 ± 8.5 mm Hg. The tourniquet was deflated immediately after termination of exercise. After 15 minutes of recovery from exercise, the tourniquet was inflated to 320 mm Hg until there was a maximum plateau in muscle deoxygenation. TCF was measured before exercise as one-third of the distance midline from the inguinal crease to the proximal border of patella. This represented the site of tourniquet application. In HYP, the participants executed the exercise bouts in normobaric, systemic hypoxia with a F_iO_2 of 14.0%, corresponding to an altitude of approx. 3250 m.

4.7 | Muscle deoxygenation

Deoxygenation at the muscle level was measured by continuous-wave, near-infrared spectroscopy (NIRS), as described previously.[76] A pair of NIRS optodes was positioned over the distal part of the vastus lateralis muscle ~15 cm above the proximal border of patella. Optodes were fixed in a plastic spacer, which was attached to the skin by double-sided sticky discs to ensure direct contact between optodes and skin. A black bandage was placed over the optodes and around the leg for further fixation and to shield against extraneous light, and to minimize loss of transmitted near-infrared light. The interoptode distance was 40 mm. Skinfold thickness was measured between the emitter and receiver optodes using a skinfold calliper (Harpenden). Skinfold thickness (8.2 ± 2.7 mm) was less than half the distance separating the optodes. Circumference of the plastic spacer was marked on the skin using an indelible pen, and pictures were taken to ensure that optodes were placed at the same position in all trials. Light absorption signals were converted to HHb deoxygenation changes using a differential pathlength factor (DPF) calculated according to participant's age. The DPF was the same across trials for each participant. Data were acquired at 10 Hz and subsequently filtered in R software (ver. x64 3.2.5, R Foundation for Statistical Computing, USA) using a 10th order zero-lag, low-pass Butterworth filter with a cut-off frequency of 0.1. The optimal cut-off frequency (ie reducing over- and underestimation of local means) was predetermined by an iterative analysis of root-mean-squared residuals derived from the application of multiple filters by use of a range of cut-off frequencies (0.075-0.150). Filtered data were used for the final analysis. Time alignment and normalization to the signal range between baseline (resting) and maximum (full occlusion) readings were completed in Excel (Ver. 2013, MS Office, Microsoft).

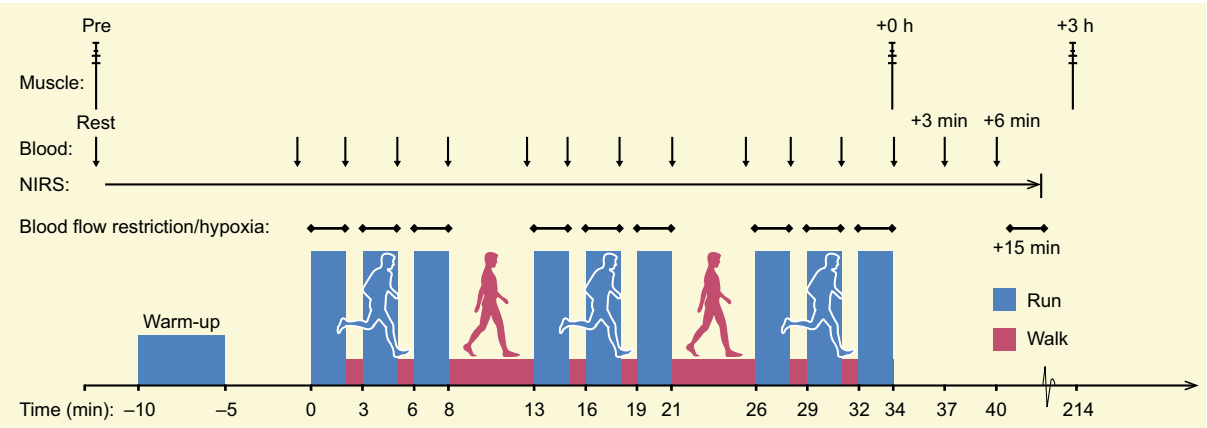

FIGURE 11 Time-aligned, schematic representation of the experimental design. The participants performed 3 exercise trials separated by 1 week consisting of running without (control) or with the muscle blood flow partially occluded (blood flow restriction, BFR), or in normobaric, systemic hypoxia (hypoxia). The exercise intensity was set according to the participants' individual lactate threshold (~12 km h^{-1}). Muscle was sampled at rest before, immediately post (+0 hour) and after 3 hours (+3 hours) of recovery from each trial. Blood was sampled from an antecubital vein at the time points indicated. BFR was induced by inflation of a tourniquet (123 ± 12 to 226 ± 24 mm Hg during exercise and 320 mm Hg post-exercise)

4.8 | Graded exercise test (GXT)

Participants completed the GXT following a light, standardized meal ~3 hours prior to arrival. The test consisted of 4-minutes runs punctuated by 1 minute of rest. The first run commenced at 5.0 km h^{-1}, and the second at 8 km h^{-1}. The speed was then increased by 1 km h^{-1} at the onset of each subsequent run until volitional exhaustion, defined as an inability to maintain the required speed. This progression in speed allowed a minimum of 7 running stages to be completed (range: 7-11). After 5 minutes of rest, participants commenced running at the speed of the last completed run, after which the speed was increased by 1 km h^{-1} per minute until volitional exhaustion. This incremental bout was performed to ascertain attainment of a maximum 30-seconds plateau in oxygen consumption. Before the test, a facemask was placed over the mouth and nose and connected to an online, gas-analysing system for measurement of inspired and expired gases. To determine LT, blood was sampled at rest and immediately after each running stage from a 20-gauge, antecubital venous catheter. The catheter was inserted at rest in a supine position on a laboratory bed at least 20 minutes prior to the test. The LT was calculated using the modified D_{max} method as it has been shown to better discriminate between individuals in comparison with other methods.[81] VO_{2max} was determined as the mean of the 2 peak consecutive 15-seconds values recorded during the test.

4.9 | Diet and activity control

Participants consumed a standardized dinner (55 kJ kg^{-1} BM; 2.1 g carbohydrate kg^{-1} BM, 0.3 g fat kg^{-1} BM, and 0.6 g protein kg^{-1} BM) and breakfast (41 kJ kg^{-1} BM; 1.8 g carbohydrate kg^{-1} BM, 0.2 g fat kg^{-1} BM, and 0.3 g protein kg^{-1} BM) 15 and 3 hours, respectively, before every main trial. They recorded their dietary pattern within 48 hours prior to each laboratory visit and were asked to replicate the same nutritional intake as per before their first exercise trial. Participants were instructed to maintain their normal dietary pattern throughout the study and were free of anti-inflammatory drugs and supplements, as well as medicine. The participants were instructed to replicate their weekly, routine physical activity throughout the study and to avoid activity beyond daily living in the 48 hours prior to each visit. In the 3-hours period from termination of exercise to the +3 hours biopsy, oral consumption was limited to ad libitum water.

4.10 | Muscle sampling

Vastus lateralis muscle biopsies were collected from the dominant leg in all trials for consistency using the Bergström needle biopsy technique with suction, amounting to 9 biopsies per participant. To minimize bleeding, the biopsy in ISC was obtained immediately after deflation of the tourniquet. In preparation for a muscle sample, a small incision was made under local anaesthesia (5 mL, 1% Xylocaine) through the skin, subcutaneous tissue and fascia of the muscle. Incisions were separated by approx. 1-2 cm in 3 parallel lines of 3. Immediately after sampling, samples were rapidly blotted on filter paper to remove excessive blood and frozen in liquid nitrogen. The samples were stored at −80°C until being analysed. The incisions were covered with sterile Band-Aid strips and a waterproof Tegaderm film dressing (3M, North Ryde, NSW, Australia).

4.11 | Blood handling and analysis

To ensure blood samples accurately represented circulating blood, ~2 mL of blood was withdrawn and discarded before sampling of approx. 2 mL of blood per sample. After being drawn, samples were placed on ice until being analysed for lactate, pH and K^+, concentrations after exercise on an ABL 800 Flex blood gas analyzer (Radiometer, Brønshøj, Denmark).

4.12 | Arterial oxygen saturation (S_aO_2)

For safety reasons, adhesive optodes were placed on the tip of the left index finger to monitor arterial oxygen saturation during the HYP trial by pulse oximetry (Nellcor N-600, Nellcor, Hayward, CA, USA). Data were recorded at rest in the standing position on the treadmill and during the final minute of each bout of running.

4.13 | RNA isolation and reverse transcription

Muscle samples were homogenized (2 × 2 minutes at 30 Hz) in ~800 μL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using an electronic homogeniser (FastPrep FP120 Homogenizer, Thermo Savant, Thermo Fisher Scientific, Waltham, MA, USA). After homogenization, the supernatant was aspirated into a new, freshly autoclaved microfuge tube containing 250 μL chloroform (Sigma-Aldrich, St. Louis, MO, USA). After few manual inversions and 5 minutes on ice, the mixture was centrifuged (15 minutes at 12 280 *g*) at 4°C. After centrifugation, the superior phase was pipetted into a new, autoclaved microfuge tube, and 400 μL 2-isopropanol alcohol (Sigma-Aldrich) and 10 μL of 5 mol L^{-1} NaCl were added. The samples were then stored at −20°C for 3 hours to precipitate the amount of RNA. After cooling, the samples were centrifuged (20 minutes at 12 280 *g*) at 4°C, and the isopropanol aspirated. The RNA pellet was rinsed with 75%

ethanol made from DEPC-treated H_2O (Invitrogen Life Sciences) and centrifuged (8 minutes at 5890 *g*) at 4°C. After pipetting off the ethanol, the pellet was resuspended in 5 μL of heated (60°C) DEPC-treated H_2O. The samples were stored at −80°C until reverse transcription. RNA purity (mean ± SD, 1.96 ± 0.24; 260 nm/280 nm) and concentration (mean ± SD, 1.317 ± 1.311 μg μL^{-1}) were determined spectrophotometrically on a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). In addition, RNA integrity was assessed in 6 randomly chosen samples on an electrophoresis station (Experion, Bio-Rad, Hercules, CA, USA) using the manufacturer's RNA analysis kit (Experion RNA StdSens) and instructions. The RNA quality indicator (RQI) of the 6 samples was (mean ± SD) 8.1 ± 0.7. One microgram of RNA per sample was reverse-transcribed into cDNA on a thermal cycler (S1000™ Thermal Cycler, Bio-Rad) using a cDNA synthesis kit (iScript RT Supermix, #1708841; Bio-Rad). The following incubation profile was used with random hexamers and oligo dTs in accordance with the manufacturer's instructions: 5 minutes at 25°C, 20 minutes at 46°C and 1 minute at 95°C. cDNA was stored at −20°C until real-time PCR.

4.14 | Real-time RT-PCR

Real-time RT-PCR was performed to determine the expression of target and reference genes. Reactions were prepared on a 384-well plate using an automated pipetting system (epMotion 5073l, Eppendorf, Hamburg, Germany). One reaction was composed of 2 μL diluted cDNA, 0.15 μL forward and reverse primer (100 μmol L^{-1} concentration), 0.2 μL DEPC-treated H_2O and 2.5 μL iTaq universal SYBR Green Supermix (#1725125; Bio-Rad). Real-time RT-PCR was performed on a QuantStudio 7 Flex Real-Time PCR System (#4485701, Thermo Fisher Scientific) using the following protocol: denaturing at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. Reactions were run in duplicate on the same plate with 4 template-free and 4 RT-negative controls. To account for variations in input RNA amounts and the efficiency of reverse transcription, target mRNA transcript levels were normalized to the geometric mean of 3 housekeeping genes using the $2^{-\Delta\Delta C_T}$ method.[82] This correction has been shown to yield reliable and valid mRNA data.[83] Reference genes used were *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *TATA-binding protein* (*TBP*) and β*2 microglobulin* (β*2M*). The mean (±SD) coefficient of variation (CV) of duplicate reactions (cycle threshold, C_T), along with the forward and reverse sequences for the primers, is shown in Table 2. Criteria and procedure for the design of primers for NKA isoforms are presented elsewhere.[11] Primers for PGC-1α isoforms were identical to those previously used.[18] Primer specificity was confirmed by performing a melt curve analysis at the end of each PCR run. The sample size for mRNA content was n = 8 for CON, n = 6 for BFR and n = 5 for HYP. Data points were excluded if contaminated ($C_T > 35$, n = 4) or if unavailable due to a missed biopsy at +3 hours (n = 1). Limited amount of muscle precluded us from re-analysing contaminated samples.

4.15 | Dissection and fibre typing of muscle fibres

All chemicals used for dot blotting and Western blotting were from Bio-Rad unless otherwise stated. Antibodies are detailed in Table 3.

One part of each muscle biopsy (50 ± 10 mg w.w.) was freeze-dried for 40 hours, yielding 11.6 ± 2.7 mg d.w. muscle tissue. From these freeze-dried portions, a minimum of 40 single-fibre segments per sample (range: 40-120; total n = 2750) were isolated under a dissecting microscope using fine jeweller's forceps. The segments were placed in individual microfuge tubes and incubated for 1 hour at room temperature in 10-μL denaturing buffer (0.125 mol L^{-1} Tris-HCl, 10% glycerol, 4% sodium dodecyl sulphate, 4 mol L^{-1} urea, 10% mercaptoethanol and 0.001% bromophenol blue, pH 6.8), in accordance with previous procedure.[84] The denatured segments were stored at −80°C until future use.

Fibre type of individual segments was determined using dot blotting, as recently described (D Christiansen, MJ MacInnis, E Zacharewicz, BP Frankish, H Xu, RM Murphy, unpublished data). In brief, two 0.45-μm PVDF membranes were activated in 95% ethanol and equilibrated for 2 minutes in cold transfer buffer (25 mmol L^{-1} Tris, 192 mmol L^{-1} glycine, pH 8.3, 20% methanol), after which a 1.5-μL aliquot of denatured sample, corresponding to one-seventh of a fibre segment, was spotted onto each membrane. The membranes were placed at room temperature on a dry piece of filter paper to dry completely (5-10 minutes), after which they were reactivated in the ethanol and re-equilibrated in transfer buffer. After a quick wash in Tris-buffered saline-Tween (TBST), membranes were blocked in 5% non-fat milk in TBST (blocking buffer) for 5-15 minutes. One of the blocked membranes was incubated (1 in 200 in 1% BSA with PBST) with myosin heavy chain I (MHCI) antibody, and the other membrane with myosin heavy chain IIa (MHCIIa) antibody for 2 hours at room temperature with gentle rocking. After a quick wash in blocking buffer, membranes were incubated (concentration: 1:20 000) with goat anti-mouse IgG (MHCIIa, #PIE31430, ThermoFisher Scientific) or IgM (MHCI, #sc-2064, Santa Cruz Biotechnology, Santa Cruz, CA, USA) horseradish peroxidase (HRP)-conjugated

TABLE 2 Forward and reverse primer sequences used in real-time PCR, their amplification efficiency and the coefficient of variation (CV) of duplicates

Gene	Forward sequence	Reverse sequence	Efficiency	CV (%) Mean ± SD
Na^+,K^+-ATPase				
α_1	CGACAGAGAATCAGAGTGGTGT	GCCCTGTTACAAAGACCTGC	1.79	0.7 ± 0.6
α_2	ACATCTCCGTGTCTAAGCGG	AGCCACAGGAGAGCTCAATG	2.25	0.7 ± 0.5
α_3	ACTGAGGACCAGTCAGGGAC	CCTTGAAGACAGCGCGATTG	–	3.4 ± 2.4
β_1	CTGACCCGCCATCGCC	TAGAAGGATCTTAAACCAACTGCC	1.76	0.5 ± 0.4
β_2	TTCGCCCCAAGACTGAGAAC	AGAGTCGTTGTAAGGCTCCA	1.83	1.4 ± 1.0
β_3	TCATCTACAACCCGACCACC	GAAGAGCAAGATCAAACCCCAG	1.90	0.8 ± 0.6
FXYD1	AGCGAGCAGAATTCCTCCAG	GCAGGGACTGGTAGTCGTAAG	1.97	1.4 ± 1.6
PGC-1α				
Total	CAGCCTCTTTGCCCAGATCTT	TCACTGCACCACTTGAGTCCAC	2.04	0.7 ± 0.5
1α1	ATGGAGTGACATCGAGTGTGCT	GAGTCCACCCAGAAAGCTGT	2.03	1.0 ± 1.1
1α4	TCACACCAAACCCACAGAGA	CTGGAAGATATGGCACAT	2.56	0.9 ± 0.8
Oxidative stress				
Catalase	CTCAGGTGCGGGCATTCTAT	TCAGTGAAGTTCTTGACCGCT	1.90	1.2 ± 1.0
HSP70	GGGCCTTTCCAAGATTGCTG	TGCAAACACAGGAAATTGAGAACT	1.92	0.9 ± 0.5
Housekeeping				
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA	2.12	1.0 ± 0.4
β2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT	2.08	0.9 ± 0.3
TBP	CAGTGACCCAGCAGCATCACT	AGGCCAAGCCCTGAGCGTAA	2.24	1.0 ± 0.7

Note that the primer set for the *Na^+,K^+-ATPase α_3* (like several other sets we and other labs have tested) results in C_T values of ~34 (ie upper end of detection range). This is likely due to the low expression of this isoform in human skeletal muscle.

secondary antibody for 1 hour at room temperature with rocking. Membranes were then quickly rinsed in TBST, exposed to Clarity enhanced chemiluminescence reagent (Bio-Rad) and imaged on a ChemiDoc MP (Bio-Rad). The membrane incubated with MHCIIa antibody was reprobed with MHCIIx antibody for 2 hours with rocking at room temperature, after which it was exposed to the same secondary antibody as MHCI (#sc-2064, Santa Cruz Biotechnology) for 1 hour at room temperature and imaged accordingly. The difference in the host immunoglobulin species of the MHCIIa (IgG) and MHCIIx (IgM) antibodies allowed both isoforms to be quantified on the same membrane.

The remainder of each denatured fibre segment (7 μL) was grouped according to MHC expression to form samples of type I (MHCI) and type II (MHCIIa) fibres for each biopsy, in line with previous procedure.[85] The number of fibre segments included in each group of muscle fibres per biopsy was (mean ± SD) n = 12 ± 6 (range: 5-27) for type I, and n = 16 ± 5 (range 7-33) for type IIa, fibres. Hybrid fibres (expressing multiple MHC isoforms) and type IIx fibres (classified by the absence of MHCI and MHCIIa, but the presence of MHCIIx protein), both constituting 3.1% of the total pool of fibres, were excluded from analysis.

4.16 | Immunoblotting

Fibre type-specific protein abundance and phosphorylation status of AMPK and CaMKII, and their downstream targets Acetyl-CoA carboxylase (ACC) and phospholamban (PLB), respectively, and the protein content of heat-shock protein 27 (HSP27) were determined by Western blotting. Fifteen micrograms of protein per sample (~5 μL) were separated (45 minutes at 200 V) on 26 wells, 4-15% Criterion TGX stain-free gels (Bio-Rad). Each gel was loaded with all samples from one participant, 2 calibration curves (a 4- and a 3-point) and 2 protein ladders (PageRuler, Thermo Fischer Scientific). Calibration curves were of human whole-muscle crude homogenate with a known protein concentration, which was predetermined as described previously.[7] After electrophoresis, gels were UV activated (5 minutes) on a Criterion stain-free imager (Bio-Rad). Proteins were wet-transferred to 0.45 μm nitrocellulose membrane (30 minutes at 100 V) in a circulating bath at 4°C in transfer buffer (25 mmol L^{-1} Tris, 190 mmol L^{-1} glycine and 20%

TABLE 3 Primary antibodies used for dot blotting and Western blotting

Protein	Primary antibody and supplier	Host species and isotype (antibody type)	Concentration	Molecular mass (kDa)
HSP27	Thermo Fisher Scientific (#MA3-015)	Mouse IgG (monoclonal)	1:1000	~27
p-ACC Ser^{79}	Cell Signaling Technology (#3661S)	Rabbit IgG (polyclonal)	1:1000	~257
ACC	Cell Signaling Technology (#3676S)	Rabbit IgG (monoclonal)	1:1000	~257
p-AMPK-α Thr^{172}	Cell Signaling Technology (#2535)	Rabbit IgG (monoclonal)	1:500	~63
AMPK-α	Cell Signaling Technology (#2603)	Rabbit IgG (monoclonal)	1:1000	~63
p-CaMKII Thr^{287}	Cell Signaling Technology (#12716)	Rabbit IgG (monoclonal)	1:1000	~50-80
CaMKII	Cell Signaling Technology (#4436)	Rabbit IgG (monoclonal)	1:1000	~50-80
p-PLB Ser^{16}	Merck Millipore (#07-052)	Rabbit IgG (polyclonal)	1:2000	~25
PLB	Abcam (#ab2865)	Mouse IgG (monoclonal)	1:1000	~25
MHC I	Developmental Studies Hybridoma Bank, University of Iowa (#A4.840)	Mouse, IgM (monoclonal)	1:200	~200
MHC IIa	Developmental Studies Hybridoma Bank, University of Iowa (#A4.74)	Mouse, IgG (monoclonal)	1:200	~200
MHC IIx	Developmental Studies Hybridoma Bank, University of Iowa (#6H1)	Mouse, IgM (monoclonal)	1:100	~200

Antibodies were diluted in 1% bovine serum albumin in 1× phosphate-buffered saline with 0.02% sodium azide and 0.025% Tween.

methanol). Membranes were then incubated (10 minutes) in antibody extender solution (Pierce Miser, Pierce, Rockford, IL, USA), washed in double-distilled H_2O and blocked for 2 hours in blocking buffer (5% non-fat milk in Tris-buffered saline-Tween, TBST) at room temperature with rocking. To allow multiple proteins to be quantified on the same membrane, the membranes were cut horizontally at appropriate molecular masses using the 2 protein ladders as markers prior to probing with the primary antibodies overnight at 4°C, and for 2 hours at room temperature with constant, gentle rocking. Antibody details are presented in Table 3. Primary antibodies were diluted in 1% bovine serum albumin (BSA) in phosphate-buffered saline with 0.025% Tween (PBST) and 0.02% NaN_3. After washing in TBST and probing with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins; Pierce) for 1 hour with rocking at room temperature, chemiluminescent images of membranes were captured on a ChemiDoc Touch (Bio-Rad), followed by densitometry using Image Lab (Ver. 5.2.1, Bio-Rad). Protein ladders were captured under white light prior to chemiluminescent imaging without moving the membranes. Band densities for proteins were quantified by reference to the mean of the 2 linear calibration curves loaded on the same gel and normalized to the total amount of protein in each lane on the stain-free gel image. The same researcher with substantial experience with the techniques was responsible for performing all muscle analyses.

4.17 | Muscle metabolites

A portion of each freeze-dried muscle sample (2 mg d.w.) was dissected free of connective tissue, blood and fat before being powdered using a Teflon pestle. The content of ATP, PCr, creatine (Cr) and lactate in each sample was extracted using pre-cooled perchloric acid/EDTA and $KHCO_3$, and analysed fluorometrically using a modification of a method previously described,[86] where samples are analysed in a 96-well plate format. All samples from each participant, along with 2 standards of either ATP, PCr, Cr or lactate, a 4-point NADH standard curve and blanks (ie double-distilled H_2O), were analysed in triplicate on the same plate. Absorbance readings of samples were normalized to the standards and subtracted blanks. The content of PCr, Cr and ATP was adjusted to Cr level across trials.

4.18 | Statistics

Data were firstly assessed for normality using the Shapiro-Wilk test. An appropriate transformation of data was applied, if necessary, to obtain a normal distribution prior to subsequent statistical analyses. Paired Student's t tests were applied to test the null hypotheses of no effects of time (Pre, +3 hours) within condition using the $2^{-\Delta\Delta C_t}$ expression data, and to test for differences between conditions (CON, BFR, HYP), using the ΔmRNA values (ie difference between Pre and +3 hours). For blood and metabolite data,

a 2-way repeated-measures (RM) ANOVA was used to test the null-hypotheses of no effects of time (Pre, +3 hours) or condition (CON, BFR, HYP). The same test was used to assess the null hypotheses of no effects of time (Pre, +0 hour) or fibre type (type I and type II) within condition for the content of total and phosphorylated proteins and to evaluate conditional interactions with time (Pre, +0 hour) within fibre type. Data normalized to total protein, and not relative changes, were used for protein analyses. For the Butterworth-filtered NIRS data, a 2-way RM ANOVA was used to test the null hypothesis of no time and condition effects. Multiple pairwise, post hoc analyses used the Tukey test. Interpretation of effect size (*d*) was based on Cohen's conventions, where <0.2, 0.2-0.5, >0.5-0.8 and >0.8 were considered as trivial, small, moderate and large effect, respectively.[87] Data are reported as means ± SEM unless otherwise stated. The α-level was set at $P \leq .05$. Statistical analyses were performed in Sigma Plot (Ver. 11.0, Systat Software, San Jose, CA, USA).

ACKNOWLEDGEMENTS

We thank the participants for being engaged throughout the study and for their donations of muscle tissue. We are also grateful for the help from Jujiao Kuang with re-measuring the mRNA content in our samples. The monoclonal antibodies for the myosin heavy chain isoforms and NKA α_1 isoform were obtained from the Development Studies Hybridoma Bank (DSHB) under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. The antibodies directed against adult human MHC I and IIa isoforms (A4.840 and A4.74, respectively) were developed by Dr H. Blau, and the antibody directed against NKA α_1 (a6F) was developed by Dr D.M. Fambrough. Danny Christiansen was supported by an International Postgraduate Research Scholarship from Victoria University, Melbourne, VIC 3011, Australia.

CONFLICT OF INTEREST

The authors have no conflict of interest that relates to the content of this article.

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How to cite this article: Christiansen D, Murphy RM, Bangsbo J, Stathis CG, Bishop DJ. Increased *FXYD1* and *PGC-1α* mRNA after blood flow-restricted running is related to fibre type-specific AMPK signalling and oxidative stress in human muscle. *Acta Physiol*. 2018;223:e13045. https://doi.org/10.1111/apha.13045

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Paper II

Christiansen D, Eibye KH, Rasmussen V, Voldbye HM, Hostrup M, Gunnarsson TGP, Thomassen M, Lindskrog MS, Skovgaard C, Nyberg M, Bishop DJ, Bangsbo J (2018): Cycling with blood flow restriction improves performance and muscle K+ regulation and alters the effect of antioxidant infusion in humans. *J Physiol. 2019* Mar 7. doi: 10.1113/JP277657. [Epub ahead of print]. Impact factor = 5.037.

Paper III

Christiansen D, David J. Bishop, James R. Broatch, Jens Bangsbo, Michael J. McKenna, Robyn M. Murphy (2018): Cold-water immersion after training sessions: effects on fiber type-specific adaptations in muscle K+ transport proteins to sprint-interval training in men. *J Appl Physiol* (1985). 2018 Aug 1;125(2):429-444. doi: 10.1152/japplphysiol.00259.2018. Impact factor = 3.256

J Appl Physiol 125: 429–444, 2018.
First published May 10, 2018; doi:10.1152/japplphysiol.00259.2018.

RESEARCH ARTICLE

Cold-water immersion after training sessions: effects on fiber type-specific adaptations in muscle K^+ transport proteins to sprint-interval training in men

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[1]*Institute for Health and Sport, Victoria University, Melbourne, Victoria, Australia;* [2]*Department of Nutrition, Exercise, and Sports, University of Copenhagen, Copenhagen, Denmark;* [3]*School of Medical and Health Sciences, Edith Cowan University, Perth, Western Australia, Australia; and* [4]*Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia*

Submitted 22 March 2018; accepted in final form 3 May 2018

Christiansen D, Bishop DJ, Broatch JR, Bangsbo J, McKenna MJ, Murphy RM. Cold-water immersion after training sessions: effects on fiber type-specific adaptations in muscle K^+ transport proteins to sprint-interval training in men. *J Appl Physiol* 125: 429–444, 2018. First published May 10, 2018; doi:10.1152/japplphysiol.00259.2018.—Effects of regular use of cold-water immersion (CWI) on fiber type-specific adaptations in muscle K^+ transport proteins to intense training, along with their relationship to changes in mRNA levels after the first training session, were investigated in humans. Nineteen recreationally active men (24 ± 6 yr, 79.5 ± 10.8 kg, 44.6 ± 5.8 ml·kg^{-1}·min^{-1}) completed six weeks of sprint-interval cycling, either without (passive rest; CON) or with training sessions followed by CWI (15 min at 10°C; COLD). Muscle biopsies were obtained before and after training to determine abundance of Na^+,K^+-ATPase isoforms (α_{1-3}, β_{1-3}) and phospholemman (FXYD1) and after recovery treatments (+0 h and +3 h) on the first day of training to measure mRNA content. Training increased ($P < 0.05$) the abundance of α_1 and β_3 in both fiber types and β_1 in type-II fibers and decreased FXYD1 in type-I fibers, whereas α_2 and α_3 abundance was not altered by training ($P > 0.05$). CWI after each session did not influence responses to training ($P > 0.05$). However, α_2 mRNA increased after the first session in COLD (+0 h, $P < 0.05$) but not in CON ($P > 0.05$). In both conditions, α_1 and β_3 mRNA increased (+3 h; $P < 0.05$) and β_2 mRNA decreased (+3 h; $P < 0.05$), whereas α_3, β_1, and FXYD1 mRNA remained unchanged ($P > 0.05$) after the first session. In summary, Na^+,K^+-ATPase isoforms are differently regulated in type I and II muscle fibers by sprint-interval training in humans, which, for most isoforms, do not associate with changes in mRNA levels after the first training session. CWI neither impairs nor improves protein adaptations to intense training of importance for muscle K^+ regulation.

NEW & NOTEWORTHY Although cold-water immersion (CWI) after training and competition has become a routine for many athletes, limited published evidence exists regarding its impact on training adaptation. Here, we show that CWI can be performed regularly without impairing training-induced adaptations at the fiber-type level important for muscle K^+ handling. Furthermore, sprint-interval training invoked fiber type-specific adaptations in K^+ transport proteins, which may explain the dissociated responses of whole-muscle protein levels and K^+ transport function to training previously reported.

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cold-water immersion; fiber type; FXYD1; Na^+,K^+-ATPase; single-fiber western blotting; training

INTRODUCTION

Submersion into cold water (≤15°C) after training and competition has become a routine for many athletes, such as those engaged in cycling and football. Cold-water immersion (CWI) in recovery has been shown to reduce the decline in maximal force (8) and intense exercise performance (40) invoked by prior intense exercise, although effects of CWI were absent (10, 61) or even detrimental to physical recovery (9) in other studies. While the impact of CWI in recovery on subsequent physical performance is well studied, few studies have examined its effects on training adaptation. CWI in recovery from each training session reduced increases in muscle strength, mass, and anabolic signaling after resistance training (62) but had no effect on adaptations in muscle mitochondrial content and 2-km cycling time-trial performance after sprint-interval training in recreationally active men (7). In contrast, enhancements in muscle capillary density after resistance training were found when each training session was followed by CWI (16). Thus, effects of CWI on training adaptation appear to be dependent on training mode and/or the component of muscle function studied.

During brief (<6 min) exhaustive exercise, potassium ions (K^+) accumulate in the muscle interstitium, which has been linked to impairment of muscle force development caused by cell membrane depolarization (41) and/or reduced central motor drive (1, 29). Interstitial K^+ accumulation is primarily counteracted by activation of the Na^+,K^+-ATPase, which promotes cellular Na^+ efflux and K^+ influx in a 3:2 ratio (12). The Na^+,K^+-ATPase is thus an important system for maintaining ion homeostasis in the musculature and performance during intense exercise. In agreement, concomitant increases in Na^+,K^+-ATPase-isoform abundance, muscle K^+ regulation, and intense exercise performance have been observed after intense training (52). However, effects of CWI recovery on intense training-induced adaptations in K^+ regulatory systems, such as the Na^+,K^+-ATPase, remain to be examined in humans.

Exposure to cold has been shown to increase the production of reactive oxygen species (ROS) (64) and norepinephrine

(22), which has been linked to greater oxidative stress (28), whereas substantial ROS production (and resultant oxidative stress) is known to be one factor responsible for increases in Na^+,K^+-ATPase mRNA expression in human skeletal muscle (11, 47). Furthermore, CWI may increase muscle nonshivering thermogenesis in some individuals, causing an increase in cytosolic calcium concentration (4), which has been linked to elevated Na^+,K^+-ATPase α-isoform mRNA content in rat muscles (55). Given that steady-state protein abundance is largely determined by variance in mRNA level (35), this evidence indicates that CWI may be a potent stimulus to augment adaptations in Na^+,K^+-ATPase content to intense training in humans. However, this has not been examined. Furthermore, the relationship between changes in mRNA and protein content for Na^+,K^+-ATPase isoforms to training remains to be studied in the same individuals.

In skeletal muscle, an active Na^+,K^+-ATPase is composed of a catalytic α subunit, a structural β subunit, and an accessory subunit, phospholemman (FXYD) (13). Each of these subunits is expressed as different isoforms in type I and II muscle fibers in humans ($\alpha_1\alpha_{1-3}$, β_{1-3}, and FXYD1) (69, 75). In rats, fiber type-specific differences in the capacity for Na^+ and K^+ transport have been related to a different expression of Na^+,K^+-ATPase isoforms (31). Despite this evidence, only three studies have assessed the impact of training on Na^+,K^+-ATPase-isoform abundance at the fiber-type level in humans. In one of these studies, intense aerobic training elevated α_2 abundance in type II fibers (51%) in older adults (74). In another study, higher abundance of β_1 (33%) and FXYD1 (108%) in type-I and α_1 (51%) and FXYD1 (60%) in type-II fibers was reported after training with restricted muscle blood flow compared with training with the circulation intact (Christiansen et al., unpublished observations). In contrast, 4 wk of sprint-interval training had no effect on α-isoform abundance (α_1, α_2 and α_3) in type I and II fibers (75), although these isoforms have been reported to change in whole-muscle samples in response to a substantially (~12–18 times) greater training volume (5, 26, 43). Thus, the duration of training was likely too short to facilitate changes in abundance of Na^+,K^+-ATPase isoforms in the study by Wyckelsma et al. (75). Thus, effects of anaerobic training modalities (e.g., sprint-interval) defined as brief (<30 s), maximal exercise bouts performed over >1 day on fiber type-dependent expression of Na^+,K^+-ATPase isoforms in humans are presently unclear.

Thus, the primary aim of the present study was to evaluate the effects of 6 wk of sprint-interval training with or without CWI in recovery from each training session on Na^+,K^+-ATPase-isoform abundances (α_{1-3}, β_{1-3}, and FXYD1) in type I and II muscle fibers in men. To provide insights into the relationship between changes in mRNA and protein, the second aim of the study was to examine the effects of a single session of sprint-interval exercise on muscle mRNA content of Na^+,K^+-ATPase isoforms in the same human cohort. We hypothesized that CWI would promote adaptations in α-isoform abundance to the training intervention.

MATERIALS AND METHODS

Ethical approval. This study was approved by the Human Research Ethics Committee of Victoria University (Melbourne, Australia; HRE12–335) and conformed to the latest version of the Declaration of Helsinki. The participants received a detailed oral and written plain-language explanation of the procedures, potential risks, and benefits associated with the study before providing oral and written consent. Rat and mouse tissue was obtained from animals used under La Trobe University Animal Ethics Committee (approval no. AEC 14–33).

Participants. Nineteen healthy men volunteered to participate in this study. Their age, body mass, height, and maximal oxygen uptake ($\dot{V}O_{2max}$) were (mean ± SD) 24 ± 6 yr, 79.5 ± 10.8 kg, 180.5 ± 10.0 cm, and 44.6 ± 5.8 $ml \cdot kg^{-1} \cdot min^{-1}$, respectively. The participants were nonsmokers and engaged in physical activity several days per week but were neither specifically nor highly trained.

Experimental design. Before the first biopsy session, participants reported to the laboratory on two separate occasions to be accustomed to the exercise protocol and recovery treatments. During this period, they also performed a graded exercise test to volitional exhaustion on an electromagnetically braked cycle ergometer (Lode BV, Groningen, The Netherlands), and their $\dot{V}O_{2max}$ was assessed in accordance with published methods (8). These visits were concluded at least 3 days before the first biopsy session and separated by a minimum of 24 h. We utilized a parallel two-group longitudinal study design (Fig. 1). Participants were matched on their predetermined $\dot{V}O_{2max}$ and randomly assigned by a random-number generator (Microsoft Excel, MS Office 2013, Redmond, WA) in a counter-balanced fashion to one of two recovery treatments: CWI (COLD, $n = 9$) or nonimmersion rest at room temperature (CON, $n = 10$). The assigned recovery protocol was performed upon completion of an intense sprint-interval exercise protocol. A muscle biopsy was obtained at rest before exercise (Pre), and 2 min (+0 h) and 3 h (+3 h) after the allocated recovery treatment. These samples were used to quantify mRNA expression. After this session, participants performed the same type of exercise three times per week for 6 wk, with each session followed by their assigned recovery treatment. Approximately 48–72 h after the last training session, another biopsy was obtained at rest (Post). Resting samples obtained before and after training were used to determine protein abundance in type I and II fibers. This study was part of a

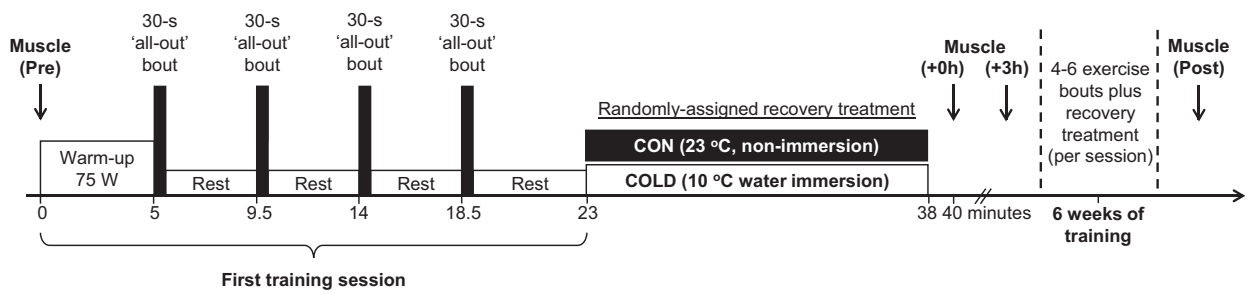


Fig. 1. Time-aligned illustration of the experiment. Muscle was sampled at rest before exercise (Pre), 2 min post (+0 h), and 3 h after (+3 h) 15 min of passive rest at room temperature (CON group) or cold-water immersion up to the umbilicus (~10°C; COLD group) that followed the first sprint-interval session. A final biopsy was sampled at rest 48 to 72 h after the last training session (Post) of 6 wk of sprint-interval training either without (CON) or with cold-water immersion (COLD) after each training session.

larger research project investigating the effects of postexercise CWI on muscle adaptation in humans. The complementary data is reported elsewhere (7). Because of insufficient size of some muscle biopsies allocated to this part of the study, protein abundance was determined in 12 of these participants (CON, $n = 7$; COLD, $n = 5$).

Exercise protocol and training. Experiments and training sessions took place in the Exercise Physiology Laboratory at the Institute for Health and Sport (Victoria University, Melbourne, Australia). The participants completed the first biopsy session on the same electrically braked cycle ergometer as that used during the familiarization. After a 5-min warm-up at a constant absolute intensity (75 W), the participants performed four 30-s maximal intensity ("all out") sprint efforts at a constant relative flywheel resistance of 7.5% of body mass, interspersed by 4 min of passive recovery in which they remained seated with their legs resting on the pedals. Each effort was commenced from a flying start at ~120 rotations/min, brought about by the investigators' manual acceleration of the flywheel before each effort. The participants remained seated in the saddle throughout the entire session. Augmented verbal feedback was provided to the participants by one investigator in a consistent manner throughout each effort. This protocol was repeated in every training session and was performed under standard laboratory conditions (~23°C, ~35% relative humidity). Participants trained three times per week for 6 wk. To ensure a progressive physiological stimulus, participants performed four sprint repetitions in *wk 1–2*, five in *wk 3–4*, and six in *wk 5–6*. Pedal resistance was modified (7.5%–9.5% of body mass) during training to ensure a minimum fatigue-induced decline in mean power output of 20 W/s.

Recovery treatments. Five minutes after termination of the sprint-interval exercise, participants commenced their designated 15-min recovery treatment, consisting of either rest in a seated posture with the legs fully extended on a laboratory bed at room temperature (~23°C, CON) or 10°C water immersion up to the umbilicus in the same position in an inflatable bath (COLD; iBody, iCool Sport, Miami, Australia). The water temperature was held constant by a cooling unit (Dual Temp Unit, iCool Sport) with constant agitation.

Muscle sampling. Muscle was sampled from the vastus lateralis muscle of the participants' right leg using a 5-mm Bergström needle with suction. In preparation, a small incision was made at the muscle belly through the skin, subcutaneous tissue, and fascia under local anesthesia (5 ml, 1% Xylocaine). In the first biopsy session, separate incisions were made for each biopsy and separated by ~1–2 cm to help minimize interference of prior muscle sampling on the mRNA response (18). The participants rested on a laboratory bed during each sampling procedure and between the second and third sampling time point. The biopsies at Pre and Post were obtained after ~30 min of rest in the supine position. Immediately after sampling, samples were rapidly blotted on filter paper to remove excessive blood and instantly frozen in liquid nitrogen. The samples were then stored at −80°C until subsequent analysis. The incisions were covered with sterile Band-Aid strips and a waterproof Tegaderm film dressing (3M, North Ryde, Australia).

Dissection of single-fiber segments. Muscle fiber segments were collected and prepared for Western blotting as previously described (50). Approximately 105 (range: 17–218) mg wet wt of muscle were freeze-dried for 28 h, providing 24 (3.4–49.0) mg dry wt of muscle for dissection of individual fibers. By use of fine jeweler's forceps, a minimum of 20 single-fiber segments were separated from each biopsy sample in a petri dish under a light microscope at room temperature (~45–60 min per biopsy). A camera (Moticam 2500, Motic Microscopes) attached to a monitor was connected to the microscope to manually measure, by use of a ruler, the length of each fiber segment. The mean segment length was 1.5 (range 1.0–3.2) mm, and a total of 520 fiber segments were collected. After dissection, segments were placed in individual microfuge tubes with the use of forceps and incubated for 1 h at room temperature in 10 μl SDS buffer (0.125 M Tris-HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol, and 0.001% bromophenol blue, pH 6.8) then stored at −20°C until they were analyzed.

Preparation of muscle fiber pools. One half of each solubilized fiber segment was used to qualitatively determine fiber type by Western blotting with antibodies against myosin heavy chain (MHC) I and II (see *Immunoblotting*). The other half of each fiber segment was grouped with other fiber segments from the same biopsy according to MHC expression to form samples of type I or II fibers from each biopsy, similar to the procedure described previously (30). The mean ± SD number of fiber segments analyzed per participant was 36 ± 3 (427 segments in total). The number of fiber segments included in each pool of fibers per biopsy before and after training, respectively, was 8 (range 4–14) and 5 (range 2–10) for type I fibers and 10 (range 6–13) and 12 (range 7–16) for type II fibers. Hybrid fibers (i.e., expressing multiple MHC I and IIa isoforms) were excluded from analysis ($n = 22$, ~4%). Type IIx fibers, classified by a lack of MHC I and II content despite protein present in the sample, were also excluded ($n = 5$, < 1%). Some lanes on the stain-free gel were empty, indicating that no fiber was successfully transferred into the microfuge tube ($n = 8$, ~1.5%). Based on agreement between two independent researchers who conducted visual inspections of the blots, fiber segments and points on the calibration curves were excluded from analysis if their band was unable to be validly quantified because of noise on the image caused by artifacts, if they were too faint, or were saturated.

Calibration curves. To be able to compare across gels, and to ensure blot density was within the linear range of detection (51), a four-point calibration curve of whole-muscle crude homogenate with a known amount of protein was loaded onto every gel. The homogenate was prepared from an equal number ($n = 5$) of pre- and posttraining freeze-dried fiber-type heterogeneous muscle samples. The samples were manually powdered using a Teflon pestle in an Eppendorf tube and then incubated for 1 h at room temperature in SDS buffer (0.125 M Tris-HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol, and 0.001% bromophenol blue, pH 6.8). The protein concentration of the homogenate was estimated by stain-free gel electrophoresis (Bio-Rad Laboratories, Hercules, CA). The intensity of the protein bands was compared with a standard curve of mixed human muscle homogenate with a known protein concentration.

Immunoblotting. To determine MHC isoform abundance in each fiber segment, half the solubilized segment (5 μl) was loaded onto 26-well plates, 4%–15% or 4%–20% Criterion TGX Stain-Free gels (Bio-Rad Laboratories). Each gel was loaded with 20 segments from the same participant ($n = 10$ for pre- and posttraining), two protein ladders (PageRuler, Thermo Fisher Scientific, Waltham, MA) and a calibration curve. Na^+,K^+-ATPase α_{1-3}, β_{1-3}, and FXYD1 protein in the muscle fiber pools were quantified by loading 15 μg wet wt muscle per sample, along with a calibration curve, onto the same gel using the same gel type as per above. Pools of type I and II fibers from Pre and Post from the same participant were loaded onto the same gel. Gel electrophoresis was performed at 200 V for 45 min. After UV activation for 5 min on a Criterion Stain-Free Imager (Bio-Rad Laboratories), proteins in gels were wet-transferred to 0.45 μm nitrocellulose membrane for 30 min at 100 V in a circulating, ice-cooled bath using transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). The current was, on average, ~0.50–0.75 mA and did not exceed 0.95 mA. After transfer, membranes were incubated for 10 min in Pierce Miser solution (Pierce, Rockford, IL), washed five times in double-distilled H_2O, and blocked for 1.5 h in blocking buffer [5% nonfat milk in Tris-buffered saline-Tween (TBST)] at room temperature with rocking. Membranes containing fiber pools were cut at 170, 70, and 25 kDa to redetermine fiber type (MHC isoforms, ~200 kDa) and to quantify one Na^+,K^+-ATPase α isoform (~100 kDa) along with one β isoform (~50 kDa) and FXYD1 (~12 kDa) on the same membrane. Membranes were incubated with rocking overnight at 4°C (preceded or followed by 2 h at room temperature) in primary antibody diluted in 1% bovine serum albumin

in phosphate-buffered saline with 0.025% Tween and 0.02% NaN_3 at concentrations detailed in Table 1. To improve the visualization of fiber type, the top portions of each membrane (>170 kDa) were stripped between MHC IIa (first) and MHC I (second) probes for 30 min at 37°C in Western blot stripping buffer (no. 21059, Thermo Fisher Scientific). Na^+,K^+-ATPase isoforms were quantified as first probes, except for α_2 and β_3, which were quantified as second probes following quantification of α_1 and β_1, respectively. The use of different host species for α_1 (mouse) and α_2 (rabbit) and the distinct molecular bands of β_1 and β_3 allowed their quantitative assessment on the same membrane. No stripping of these membrane portions was performed. After incubation in primary antibody, membranes were washed three times in TBST and incubated for 1 h at room temperature in horseradish peroxidase-conjugated secondary antibody (goat anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins; Pierce) diluted 1:20,000 with 5% nonfat milk. After another three membrane washes in TBST, protein bands were visualized using enhanced chemiluminescence (SuperSignal West Femto, Pierce) on a ChemiDoc MP imaging system (Bio-Rad Laboratories). Quantification of bands was performed in Image Laboratory 5.2.1 (Bio-Rad Laboratories). Linearity between blot signal (density) and tissue loaded for calibration curves was established on every membrane. The same researcher was responsible for performing all Western blots included in this study.

Antibodies for immunoblotting and animal tissue. Full details for the primary antibodies are shown in Table 1. Antibodies used to detect MHC slow- (type I, no. A4.840) and fast-type (type IIa, no. A4.74) isoforms were produced using the entire immunogen sequence (MHCI and MHCII, respectively). The former antibody recognizes a C-terminus epitope, whereas the latter remains to be mapped (Developmental Studies Hybridoma Bank, Iowa City, IA). Validation of antibodies is shown in Fig. 2 and was performed with positive and negative controls using mouse [extensor digitorum longus (EDL), soleus, and brain), rat (EDL, soleus, brain, kidney, and cardiac muscles) and human (breast cancer cell lines, embryonic kidney cells, and skeletal muscle) tissues. The rats (Sprague Dawley, 6 mo) and mice were sedentary and healthy. The animals were killed by an overdose of isoflurane. Soleus and EDL muscles, brain, kidney, and heart were dissected, separately homogenized in SDS buffer, and stored at −80°C until they were analyzed.

Reliability of Western blotting. The reproducibility of Western blotting for each of the Na^+,K^+-ATPase isoforms and FXYD1 was determined from triplicate Western blots for each of the proteins and is expressed as the coefficient of variation (Fig. 3*C*). The calibration curves from whole-muscle crude homogenate, which were loaded on every gel, were used for the analysis. Protein abundance and total protein for each amount of protein loaded was determined by normalizing the density for a given loading amount to that of the slope of the calibration curves.

RNA isolation. From each biopsy, ~25 mg wet wt of muscle were added to 1 g zirconia/silica beds (1.0 mm, Daintree Scientific, Tasmania, Australia) and homogenized in 800 μl TRIzol reagent (Invitrogen, Carlsbad, CA) using an electronic homogenizer (FastPrep FP120 Homogenizer, Thermo Savant). After centrifugation (15 min at ~12,280 *g*), cell debris was removed, and the supernatant was added to 250 μl chloroform (Sigma-Aldrich, St. Louis, MO) and centrifuged (15 min at ~12,280 *g*) at 4°C. RNA was precipitated by aspirating the superior phase into a new Eppendorf tube containing 400 μl 2-isopropanol alcohol (Sigma-Aldrich) and 10 μl of 5 M NaCl. Following storage at −20°C overnight, samples were centrifuged (20 min at ~12,280 *g*) at 4°C, after which the isopropanol was aspirated. The RNA pellet was rinsed with 75% ethanol made from diethyl pyrocarbonate (DEPC)-treated H_2O (Invitrogen) and centrifuged at ~5,890 *g* for 8 min at 4°C. After aspirating off the ethanol, the pellet was resuspended in 5 μl of heated DEPC-treated H_2O. RNA concentration and purity were determined spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific) at 260 and 280 nm. The RNA yield was 1,252 ± 467 ng/μl, and the ratio of absorbance (260 nm/280 nm) was 1.78 ± 0.11. RNA was stored at −80°C until reverse transcription.

Reverse transcription. For each sample, 1 μg of RNA was transcribed into cDNA on a thermal cycler (S1000 Thermal Cycler, Bio-Rad Laboratories) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) and the following incubation profile: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The transcription was performed with random hexamers and oligo dTs in accordance with the manufacturer's instructions. cDNA was stored at −20°C until subsequent analysis.

Primers. Primers were designed using Primer BLAST (National Center for Biotechnology Information, Bethesda, MD) and are shown in Table 2. To improve the construct validity of product amplification by real-time PCR, primers were designed to target a region on the genes encoding for most splice variants and to ensure sequence homology for the target gene only. Furthermore, primers fulfilled 3′end self-complementarity <3.00, span of an exon-exon junction, and PCR product size <150 bp. The difference in maximum melting temperature between forward and reverse primers was <2°C. The β2 microglobulin gene primer set was adopted from Vandesompele et al. (73). Primer validation was performed in two steps. First, its optimal annealing temperature (i.e., resulting in the highest yield with no nonspecific amplification) was determined using gradient PCR, and the results were verified by agarose gel (2%) electrophoresis. If primers were successful, their efficiency was evaluated by real-time PCR of a 10 × cDNA dilution series. Primer efficiency was calculated from the slope of the standard curve generated from the log-transformed cDNA dilutions and corresponding cycle threshold (C_t). From these results, each reaction was designed to yield a C_t within the linear range of detection by adjusting input cDNA concentration for every gene that was quantified.

Table 1. *Primary antibodies used for quantification of protein abundance of Na^+,K^+-ATPase isoforms, FXYD1, and MHC isoforms in groups of type I or type II human skeletal muscle fibers*

Protein	Primary Antibody and Supplier	Host Species and Isotype (Antibody Type)	Concentration	Molecular Mass, kDa
Na^+,K^+-ATPase α_1	DSHB, University of Iowa (#a6F-s, lot from 8/15/13)	Mouse IgG, (monoclonal)	1:100	~100
Na^+,K^+-ATPase α_2	Merck Millipore (#07–674, lot #2444088)	Rabbit, IgG (polyclonal)	1:5,000	~105
Na^+,K^+-ATPase α_3	Thermo Fisher (#MA3–915, lot #NJ175778)	Mouse, IgG (monoclonal)	1:1,000	~105
Na^+,K^+-ATPase β_1	Thermo Fisher (#MA3–930, lot #OH178971)	Mouse, IgG (monoclonal)	1:10,000	~50
Na^+,K^+-ATPase β_2	United BioResearch, Proteintech (#22338–1-AP, lot #00022128)	Rabbit, IgG (polyclonal)	1:500	~55
Na^+,K^+-ATPase β_3	BD Biosciences (#610992, lot #20600)	Mouse, IgG (monoclonal)	1:500	~55
FXYD1	United BioResearch (Proteintech #13721–1-AP, lot #00015622)	Rabbit, IgG (polyclonal)	1:5,000	~12
MHC I	DSHB, University of Iowa (#A4.840)	Mouse, IgM (monoclonal)	1:200	~200
MHC IIa	DSHB, University of Iowa (#A4.74)	Mouse, IgG (monoclonal)	1:200	~200

DSHB, Developmental Studies Hybridoma Bank; MHC, myosin heavy chain; FXYD1, phospholemman. Antibodies were diluted in 1% bovine serum albumin in 1 × phosphate-buffered saline with 0.02% sodium azide and 0.025% Tween.

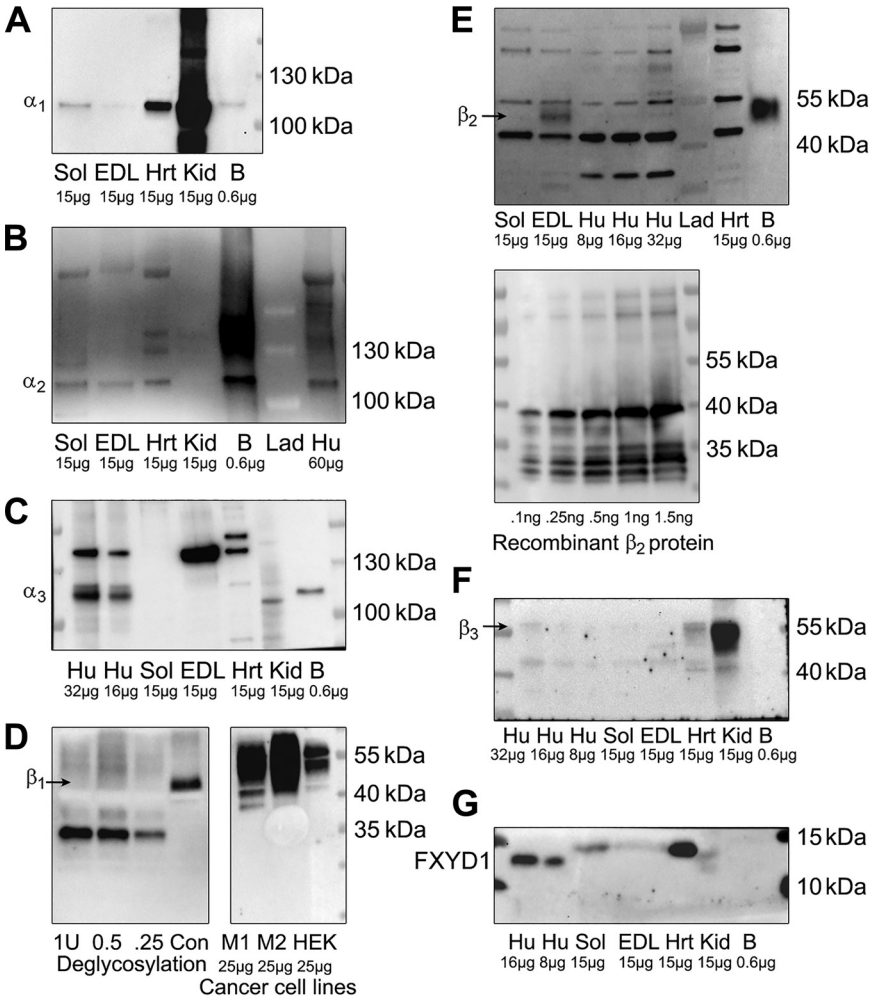


Fig. 2. Validation of antibodies used to quantify the protein abundance of Na^+,K^+-ATPase isoforms and phospholemman (FXYD1). Crude samples of human vastus lateralis (Hu) and rat skeletal muscles [extensor digitorum longus (EDL) and soleus (SOL)]; rat cardiac muscle/heart (Hrt), kidney (Kid), and brain (B); breast cancer cell lines [MDA-MB-231 (M1) and MCF10.Ca1d (M2)]; and a control cell line [human embryonic kidney (HEK) 293] were loaded onto 4%–15% gradient, Criterion stain-free gels. After SDS-PAGE, proteins were wet-transferred onto 0.45 μm nitrocellulose membrane. Membranes were incubated with antibodies raised against each of the Na^+,K^+-ATPase isoforms (α_{1-3} in *A*, *B*, and *C*; β_{1-3} in *D*, *E*, and *F*, respectively) or phospholemman (FXYD1) (*G*), posttreated with specific secondary antibodies and imaged using chemiluminescence. Isoform bands and molecular weight markers (lad) are shown in each image. In *D*, deglycosylation of human skeletal muscle samples were performed using PNGase incubation (3 h) at concentrations indicated in units (U). Recombinant β_2 protein (no. Ag17818, Proteintech) identical to the 64–171 aa derived from E.coli, PGEX-4T with N-terminal GST was used to verify the specificity of the β_2 antibody (*E*, *bottom*). See METHODS for additional details.

Real-time PCR and mRNA data treatment. mRNA expression was quantified by real-time PCR on a Mastercycler RealPlex 2 (Eppendorf, Hamburg, Germany). Each reaction (10 μl) was composed of 4 μl of diluted cDNA, 5 μl of 2 × concentrated iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) with SYBR Green I as the fluorescent agent, 0.6 μl of primer diluted in DEPC-treated H_2O, and 0.4 μl DEPC-treated H_2O. Reactions were denatured at 95°C for 3 min to activate the enzyme before PCR cycling. PCR cycles (40) were executed by heating to 95°C for 15 s followed by 60°C for 60 s. Samples and template-free controls were loaded in duplicate on the same plate, and samples from the same subject from all time points were on the same plate. Reactions were prepared using an automated pipetting system (epMotion 5070, Eppendorf). The expression of target genes was normalized to that of three housekeeping genes using the $2^{-\Delta\Delta CT}$ method (36). This correction has been shown to yield reliable and valid mRNA data (73). Housekeeping genes used were glyceraldehyde 3-phosphate dehydrogenase, TATA-binding protein, and β2 microglobulin as their mRNA expression remained unchanged with our exercise protocol ($P > 0.05$, using 2^{-Ct}; data not shown). The mean (± SD) intra assay and interassay coefficient of variation for the investigated genes are shown in Table 3. Prior to statistical analysis, an iterative elimination of outliers was performed using the following criteria: an arbitrary expression at predeviating >three-fold from the group mean at Pre. Four outliers were identified: one in CON for α_1 (5.3-fold) and one in COLD for α_1, α_2, and β_1 (4.7-, 3.1- and 3.5-fold, respectively). Thus, the sample size in CON and COLD, respectively, was $n = 9$ and $n = 8$ for α_1, $n = 10$ and $n = 8$ for α_2 and β_1, and $n = 10$ and $n = 9$ for the other genes.

Statistical analysis. Statistical analyses were performed in Sigma Plot (Version 11.0, SYSTAT, San Jose, CA). Data were assessed for normality using the Shapiro-Wilk test. An appropriate transformation was used, if required, to ensure a normal distribution of data before subsequent analysis. For mRNA data, a two-way repeated-measures ANOVA was used to evaluate the effect of group (COLD vs. CON) and time (Pre, +0 h, +3 h) using the $2^{-\Delta\Delta Ct}$ expression data. For protein data, the same test was used to evaluate the effects of time (Pre, Post) and fiber type (I and II) within group and using data from both groups (pooled data). A one-way ANOVA was used to assess the effect of group (COLD vs. CON) on Pre to Post change in protein content within fiber type (I and II). Data normalized to total protein, and not relative changes, were used for protein analysis. Where applicable, multiple pairwise post hoc comparisons used the Tukey test. Cohen's (14) conventions were adopted for interpretation of effect size (*d*), where <0.2, 0.2–0.5, >0.5–0.8 and >0.8 were considered as trivial, small, moderate, and large, respectively. Data are reported as geometric mean ± 95% confidence intervals (CI95) in figures. Note that the mRNA and protein expression at Pre is not equal to 1.0 due to the nature of using geometric means. F statistic (F) and d ± CI95 are shown for time and fiber-type effects, as well as group interactions. The α-level was set at $P \leq 0.05$.

RESULTS

In figures, individual data are displayed on the left with each symbol representing the same participant for both proteins and mRNA. On the right, geometric means ± CI95 are shown. For

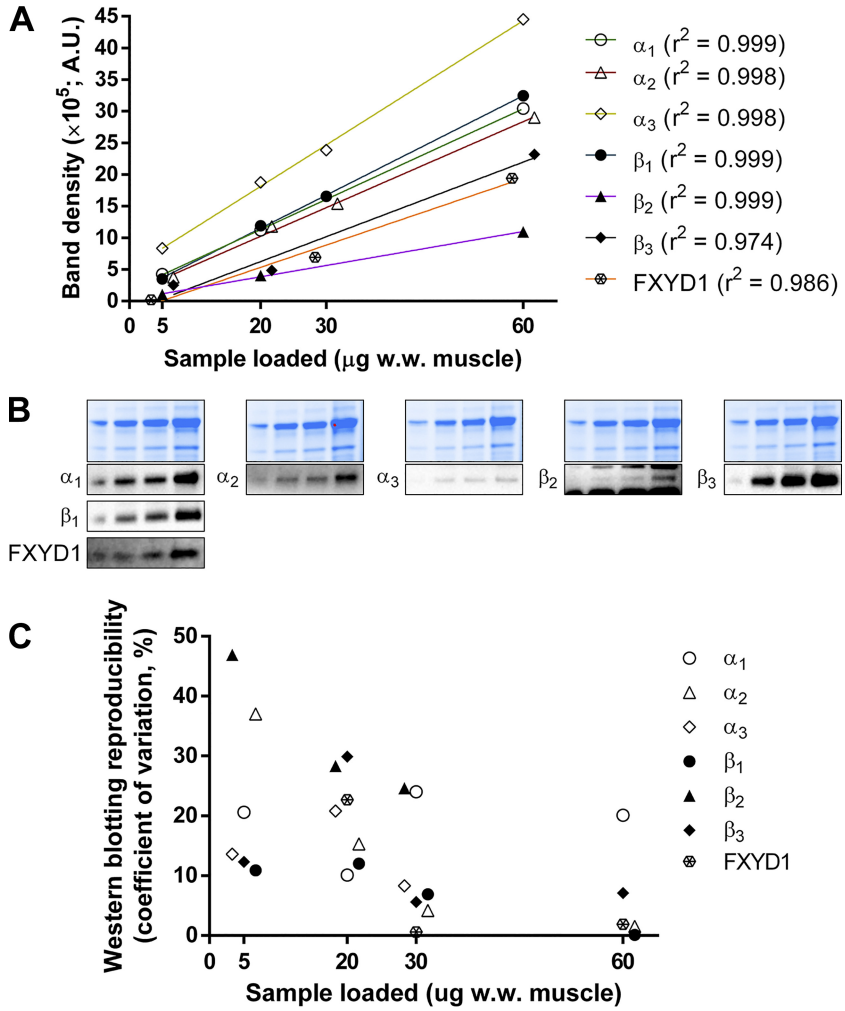


Fig. 3. Representative calibration curves (*A*) and blots (*B*) and Western blotting reproducibility (*C*) for Na^+,K^+-ATPase isoforms and phospholemman (FXYD1). For every calibration curve, 5, 20, 30, and 60 μg wet wt of human vastus lateralis muscle was loaded onto the gels. To enable visualization of all symbols, some data series in *A* and points in *B* were shifted by 1.70 units on the horizontal axis. Reproducibility was expressed as the coefficient of variation (CV) and was calculated by normalizing the density for a given loading amount to that of the slopes of the calibration curves for isoform blots and total protein on stain-free gel. In *B*, total protein on stain-free gels (*top*) and representative blots for the four-point calibration curves (*bottom*) are shown. A.U., arbitrary units.

protein data, fold-changes are reported relative to the participants' geometric mean at Pre in type I fibers in CON and for mRNA data relative to the participants' geometric mean at Pre in CON.

Validation of antibodies for immunoblotting. The results from our antibody validation are shown in Fig. 2.

The isoform specificity of the α_1 monoclonal antibody (#a6F) was verified using rat kidney and brain as positive controls (Fig. 2*A*). Our results support previous findings in similar tissues (2). We were also able to replicate the muscle type-specific distribution of α_1 observed previously in rat skeletal muscles using other α_1 antibodies (17, 37).

Specificity of the α_2 polyclonal antibody (no. 07–674) was verified by a clear band at the predicted molecular weight (105 kDa) in human and rat skeletal muscles and rat cardiac muscle and brain (Fig. 2*B*). These findings support previous results in

Table 2. *Forward and reverse primer sequences used to determine gene expression of Na^+,K^+-ATPase isoforms and reference genes, and their amplification efficiency during real-time PCR*

Gene	Forward Sequence	Reverse Sequence	Efficiency
Ion transport			
Na^+,K^+-ATPase α_1	CGACAGAGAATCAGAGTGGTGT	GCCCTGTTACAAAGACCTGC	1.8
Na^+,K^+-ATPase α_2	ACATCTCCGTGTCTAAGCGG	AGCCACAGGAGAGCTCAATG	2.3
Na^+,K^+-ATPase α_3	ACTGAGGACCAGTCAGGGAC	CCTTGAAGACAGCGCGATTG	
Na^+,K^+-ATPase β_1	CTGACCCGCCATCGCC	TAGAAGGATCTTAAACCAACTGCC	1.8
Na^+,K^+-ATPase β_2	TTCGCCCCAAGACTGAGAAC	AGAGTCGTTGTAAGGCTCCA	1.8
Na^+,K^+-ATPase β_3	TCATCTACAACCCGACCACC	GAAGAGCAAGATCAAACCCCAG	1.9
FXYD1	AGCGAGCAGAATTCCTCCAG	GCAGGGACTGGTAGTCGTAAG	2.0
Housekeeping genes			
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA	1.9
β2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT	1.8
TBP	CAGTGACCCAGCAGCATCACT	AGGCCAAGCCCTGAGCGTAA	2.0

β2M, β_2 microglobulin; FXYD1, phospholemman; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA-binding protein.

Table 3. *Intra- and interassay variability of raw C_T values for mRNA expression determined with RT-PCR*

Gene	Intraassay, %	Interassay, %
Na^+,K^+-ATPase		
α_1	3.5	2.0
α_2	9.5	4.3
α_3	3.0	2.8
β_1	3.2	2.0
β_2	4.2	2.2
β_3	3.6	1.8
FXYD1	5.3	2.6
GAPDH	8.5	4.0
TBP	6.1	2.8
β2M	11.6	5.6

Each sample was loaded in duplicate wells in the same real-time-PCR run ($n = 47$). Inter-assay CV was calculated from two separate real-time-PCR runs for the same gene. β2M, β_2 microglobulin; C_T, cycle threshold; CV, coefficient of variation; FXYD1, phospholemman; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA-binding protein.

the same tissues (37, 70, 75). We found that α_2 was absent in rat kidney, which also corroborates with the literature (15, 25, 34). Basic Local Alignment Search Tool (BLAST) analysis of the peptide sequence specific to the α_2 antibody (no. 07–674, lot no. 2444088; aa sequence 432–445, human) revealed no cross reactivity with other Na^+,K^+-ATPase isoforms.

The α_3 isoform is highly expressed in rat brain (33) but absent in rat cardiac muscle (37, 66) and kidney (25). One study has shown that α_3 may also be absent in rat skeletal muscle (33). By use of the monoclonal α_3 antibody (no. MA3–915), we support these previous findings (Fig. 2*C*). The multiple bands in human skeletal muscle could indicate multiple splice variants, as observed previously (71).

The monoclonal β_1 antibody (no. MA3–930) has been used previously to quantify β_1 protein in human muscle samples despite no clear evaluation of specificity of the lot used (49). As β_1 protein is heavily glycosylated (72) and highly expressed in human cancer cell lines (63), we tested the specificity of our β_1 antibody lot using deglycosylated (peptide:N-glycosidase (PNGase)-treated for 3 h) and control human crude skeletal muscle samples and two human breast cancer (MDA-MB-231 and MCF10.Ca1d) and control (human embryonic kidney) cell lines (Fig. 2*D*). Our findings of a substantial downshift of the predicted β_1 band in deglycosylated samples and the markedly higher density of the same band in cancer cell lines versus human embryonic kidneys strongly support the specificity of our antibody lot for β_1 protein. BLAST analysis of the peptide sequence specific to the β_1 antibody (no. MA3–930; aa sequence 195–199, sheep) confirmed an absence of cross reactivity with other Na^+,K^+-ATPase isoforms.

Specificity of the polyclonal β_2 antibody (no. 22338–1-AP) was supported by detection of recombinant β_2 protein (no. Ag17818, Proteintech; Fig. 2*E*, *bottom*). In further support, our antibody was able to detect a band at the predicted molecular weight in our positive (rat brain), but not in our negative (rat cardiac muscle), control sample. It also revealed a muscle type-specific expression of β_2 in rat (Fig. 2*E*), in line with what has been found previously (17, 32). BLAST analysis of the peptide sequence specific to the β_2 antibody lot used in the present study revealed that it did not cross react with other Na^+,K^+-ATPase isoforms.

Using the monoclonal β_3 antibody (no. 610992), we detected a band at the predicted molecular weight in human muscle (albeit weak in our whole-muscle crude homogenate), rat soleus and cardiac muscle, and kidney (Fig. 2*F*). Presence of β_3 protein in rat kidney is in coherence with the presence of β_3 gene transcripts in the same tissue (39). We found that β_3 protein was absent in rat EDL muscle and brain, suggesting tissue-specific expression at the protein level.

Specificity of the polyclonal FXYD1 antibody (no. 13721–1-AP) was supported by detection of a band at the predicted molecular weight for our positive controls (human and rat skeletal muscles, rat cardiac muscle, and kidney; Fig. 2*G*). FXYD1 is either not expressed or very lowly expressed in central nervous tissue (15). In accordance, we did not detect it in rat brain. The higher density of the band in rat soleus versus EDL muscle supports previous findings (60). The longer migration of FXYD1 in the human versus rat tissues could indicate species- or case-specific differences in post-translational modification, such as phosphorylation (19, 68). BLAST analysis of the peptide sequence specific to the FXYD1 antibody (sequence 1–92 encoded by BC032800) showed no signs of cross reactivity with Na^+,K^+-ATPase isoforms.

Western blotting technical error. The points constituting calibration curves on gels were strongly correlated ($r^2 \geq 0.98$, $n = 22$ gels, Fig. 3*A*). Representative blots for these curves are shown in Fig. 3*B*. The reproducibility of Western blotting for each of the Na^+,K^+-ATPase isoforms and FXYD1 is shown in Fig. 3*C*. The technical error for these isoforms was ~10%–30% for the protein loading amount used (1.5 fiber worth of protein). Furthermore, the error was isoform-dependent, and for most isoforms, inversely related to the amount of protein loaded on each gel (Fig. 3*C*).

Representative blots and verification of fiber type of fiber pools. Representative blots for training-induced effects on protein abundance and verification of fiber type of fiber pools are shown in Fig. 4. It is clear from these results that fibers were grouped correctly, confirmed by the clear difference in MHC-isoform expression between type I and II fiber pools.

Na^+,K^+-ATPase α_1. In CON, α_1 protein increased with training (main effect for time: $F = 19.78$; $P = 0.004$; $d = 1.41 \pm 0.77$; $n = 7$) in both type I ($P = 0.008$; $d = 1.20 \pm 0.80$) and II ($P = 0.002$; $d = 1.56 \pm 0.78$; Fig. 5*A*) fibers. Similarly, in COLD, α_1 protein increased with training (main effect for time: $F = 8.66$; $P = 0.042$; $d = 1.55 \pm 0.81$; $n = 4$) in both type I ($P = 0.053$; $d = 1.40 \pm 0.85$) and II ($P = 0.029$; $d = 1.61 \pm 0.82$) fibers. The increase in α_1 protein was not different between groups in both fiber types (type I: $P = 0.607$; $d = 0.07 \pm 0.04$; type II: $P = 0.348$; $d = 0.36 \pm 0.21$; Fig. 5*A*). In both CON and COLD, α_1 mRNA increased from Pre to +3 h ($P < 0.001$; $d = 1.28 \pm 0.50$ and 1.29 ± 0.63, respectively), with no difference between groups (group × time interaction: $F = 0.07$; $P = 0.802$; Fig. 6*A*).

Na^+,K^+-ATPase α_2. The α_2 protein remained unchanged with training in both groups (main effect for time in CON: $F = 3.65$; $P = 0.105$; $d = 0.34 \pm 0.33$; $n = 7$; and in COLD: $F = 0.69$; $P = 0.452$; $d = 0.48 \pm 0.34$; $n = 5$; Fig. 5*B*). Based on the pooled data (both groups), α_2 protein remained unchanged with training in both fiber types (type I: $P = 0.161$, $n = 12$; type II: $P = 0.112$, $n = 12$). Based on the same data, α_2 protein was 17 ± 46% more abundant in type II compared with type I fibers (main effect for fiber type: $F = 9.63$; $P = 0.010$; $d =$

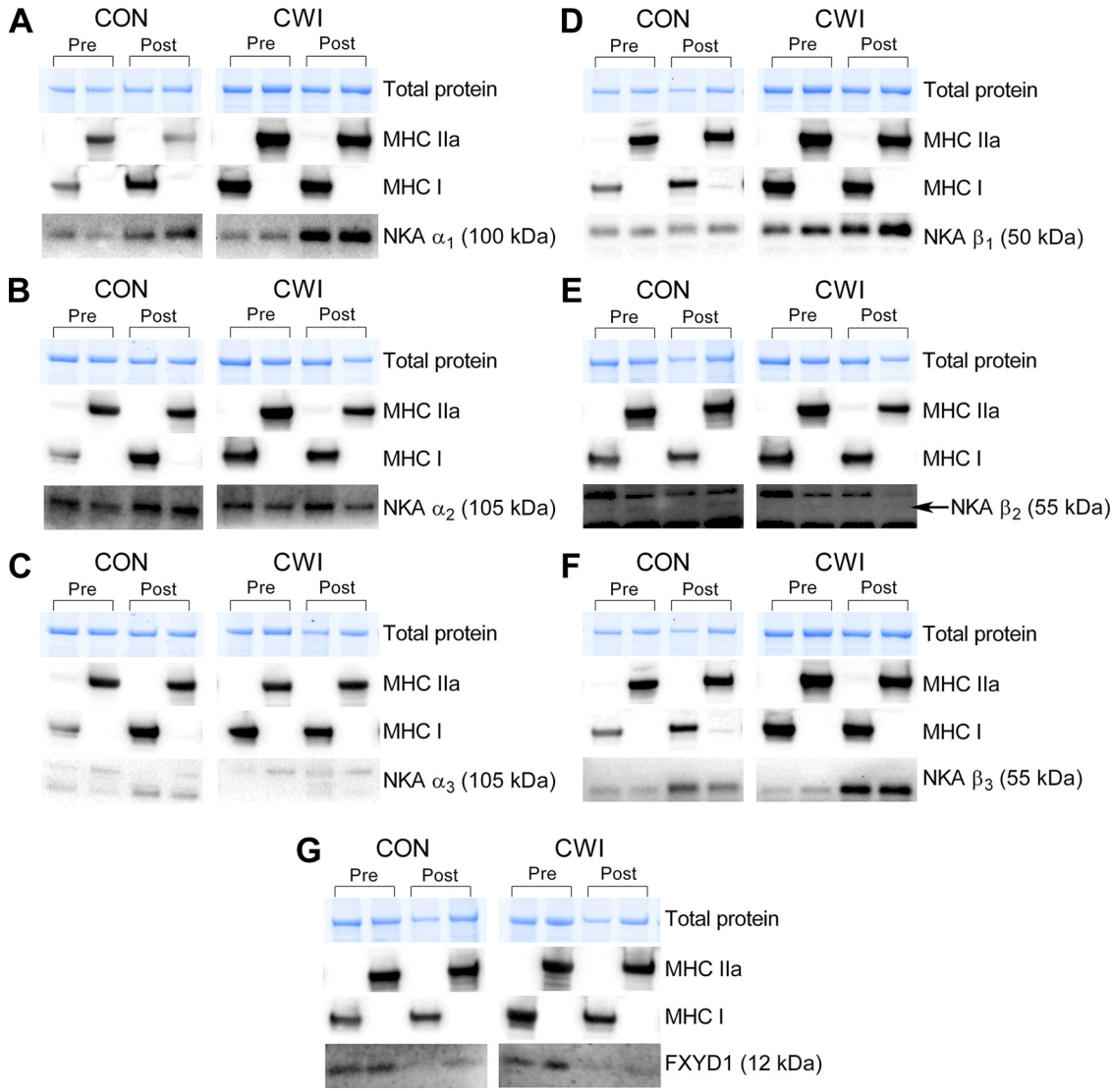


Fig. 4. Representative blots for training effects on Na^+,K^+-ATPase (NKA) isoform and phospholemman (FXYD1) abundance. Total protein on stain-free gels used for analysis (*top*), myosin heavy chain (MHC) isoform expression of fiber pools (*middle*), and representative blots for Na^+,K^+-ATPase isoforms from the same run (*bottom*). Blots for α_1, α_2, and α_3 are shown in *A*, *B*, and *C*, respectively. Blots for β_1, β_2, β_3, and FXYD1 in are shown in *D*, *E*, *F* and *G*, respectively. CON, without CWI; CWI, cold-water immersion.

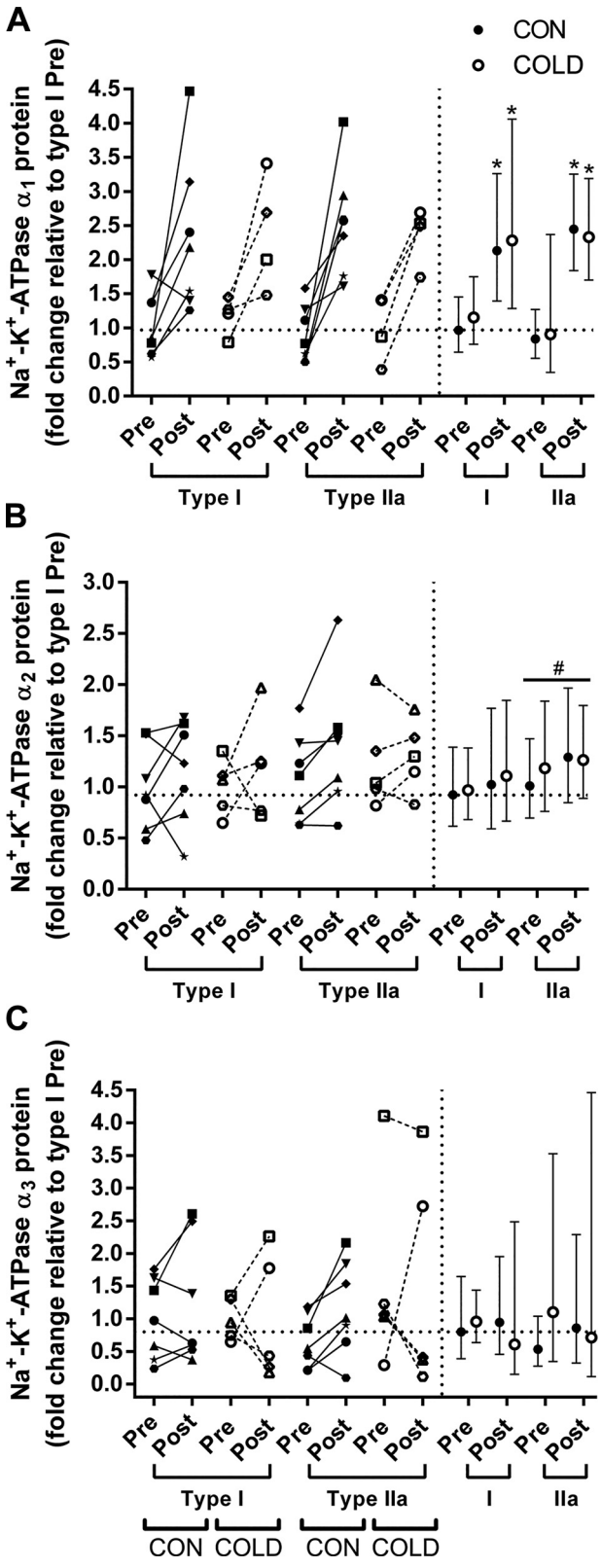
0.38 ± 0.26; $n = 12$). In COLD, α_2 mRNA increased from Pre to +0 h ($P = 0.013$; $d = 1.2 \pm 0.9$). In contrast, it remained unchanged in CON ($P = 0.862$; $d = 0.20 \pm 0.31$; Fig. 6*B*). This change in COLD was higher than in CON (group × time interaction: 1.42 ± 1.32-fold; $P < 0.001$, $d = 1.23 \pm 0.63$; Fig. 6*B*).

Na^+,K^+-ATPase α_3. The α_3 protein remained unchanged in both groups with training (main effect for time in CON: $F = 3.71$; $P = 0.103$; $d = 0.53 \pm 0.52$; $n = 7$; and in COLD: $F = 0.55$; $P = 0.501$; $d = 0.03 \pm 1.02$; $n = 5$; Fig. 5*C*). In both groups, α_3 mRNA content remained unchanged following the first training session ($F = 0.68$; $P = 0.513$; pooled $d = 0.23 \pm 0.25$), with no difference between groups (group × time interaction: $F = 0.56$; $P = 0.578$; Fig. 6*C*).

Na^+,K^+-ATPase β_1. The β_1 protein remained unchanged with training in both groups alone (main effect for time in CON: $F = 1.25$; $P = 0.306$; $d = 0.37 \pm 0.45$; $n = 7$; and in COLD: $F = 0.01$; $P = 0.960$; $d = 0.10 \pm 0.58$; $n = 5$; Fig. 7*A*). Based on the pooled data (both groups), β_1 protein increased by 44 ± 75% in type II fibers with training ($P = 0.038$; $d = 0.53 \pm 0.35$) but remained unchanged in type I fibers ($P = 0.149$; $d = 0.29 \pm 0.24$). Using the same data, β_1 protein was 62 ± 80% more abundant in type II than in type I fibers at Post ($P = 0.003$; $d = 0.77 \pm 0.45$, $n = 12$). In both groups, β_1 mRNA remained unchanged following the first training session ($F = 0.36$; $P = 0.700$; pooled $d = 0.29 \pm 0.31$), with no difference between groups (group × time interaction: $F = 0.05$; $P = 0.949$; Fig. 8*A*).

Na^+,K^+-ATPase β_2. As our Western blots did not allow for quantitative assessment of training-induced effects on β_2 abundance, these data were excluded from analysis and are not presented. A representative blot is shown in Fig. 4*E*. Based on the pooled data (both groups), β_2 protein was 54 ± 95% more abundant in type II compared with type I fibers (main effect for fiber type: $F = 14.84$; $P = 0.003$; $d = 0.56 \pm 0.56$; $n = 11$). In both CON and COLD, β_2 mRNA

content decreased from Pre to +3 h ($P = 0.002$, $d = 0.86 \pm 0.21$; and $P = 0.008$, $d = 0.82 \pm 0.27$, respectively), with no difference between groups (group × time interaction: $F = 0.02$; $P = 0.980$; Fig. 8*B*).



Na^+,K^+-ATPase β_3. In CON, β_3 protein increased with training (main effect for time: $F = 38.62$; $P < 0.001$; $d = 1.23 \pm 1.05$; $n = 7$) in both type I ($P = 0.003$; $d = 1.18 \pm 0.97$) and II ($P < 0.001$; $d = 1.24 \pm 1.15$) fibers. Similarly, in COLD, β_3 protein increased with training (main effect for time: $F = 22.40$; $P = 0.009$; $d = 1.18 \pm 2.93$; $n = 5$) in both type I ($P = 0.006$; $d = 1.18 \pm 2.93$) and II ($P = 0.012$; $d = 1.21 \pm 1.92$) fibers. The increase in β_3 protein was not different between groups in either fiber type (group × time interaction: $F = 0.59$, $P = 0.459$ for type I; and $F = 0.04$, $P = 0.854$ for type II). Based on the pooled data (both groups), β_3 protein increased with training (main effect for time: $F = 64.94$; $P < 0.001$; $d = 1.13 \pm 1.19$; $n = 12$) in both type I (2.55 ± 2.39-fold; $P < 0.001$; $d = 1.07 \pm 1.35$) and II (2.22 ± 1.81-fold; $P < 0.001$; $d = 1.22 \pm 1.03$; Fig. 7*B*) fibers. In both CON and COLD, β_3 mRNA increased from Pre to +3 h ($P < 0.001$, $d = 1.23 \pm 0.57$; and $P < 0.001$, $d = 1.39 \pm 0.86$, respectively), with no difference between groups (group × time interaction: $F = 0.52$; $P = 0.598$, Fig. 8*C*).

FXYD1. The FXYD1 protein remained unchanged with training in both groups alone (main effect for time in CON: $F = 1.56$, $P = 0.258$, $d = 0.66 \pm 0.34$, $n = 7$; and in COLD: $F = 0.23$, $P = 0.654$, $d = 1.17 \pm 0.27$, $n = 5$). Based on the pooled data (both groups), FXYD1 protein decreased by $33 \pm 40\%$ in type I ($P = 0.012$; $d = 0.82 \pm 0.22$) but remained unchanged in type II ($P = 0.535$; $d = 0.51 \pm 0.17$) fibers. Based on the same data, FXYD1 protein was $35 \pm 35\%$ more abundant in type I compared with type II fibers at Pre (main effect for fiber type: $F = 6.31$; $P = 0.020$; $d = 1.01 \pm 0.57$; $n = 12$) but not at Post ($F = 0.79$; $P = 0.384$; $d = 0.31 \pm 0.17$; $n = 12$; Fig. 7*C*). In both groups, FXYD1 mRNA remained unchanged following the first training session ($F = 0.68$; $P = 0.512$; pooled $d = 0.45 \pm 0.51$), with no difference between groups (group × time interaction: $F = 0.05$; $P = 0.952$; Fig. 8*D*).

DISCUSSION

The novel findings of the present study were that six weeks of sprint-interval training increased the abundance of Na^+,K^+-ATPase-isoform α_1 and β_3 in type I and II fibers, β_1 in type II fibers, and decreased FXYD1 in type I fibers, whereas training had no effect on α_2 and α_3 abundance in either fiber type. Adding 15 min of CWI (10°C) after each training session did not interfere with training-induced alterations in Na^+,K^+-ATPase-isoform abundances. Furthermore, a single session of sprint-interval exercise increased the mRNA content of α_1 and β_3 and decreased that of β_2, whereas it had no impact on α_2, α_3, β_1, and FXYD1 mRNA. Application of CWI in recovery

Fig. 5. Effect of six weeks of sprint-interval training with (COLD) or without (CON) cold-water immersion recovery on Na^+,K^+-ATPase α-isoform abundance in type I and II muscle fibers in men. α_1 (*A*), α_2 (*B*), and α_3 (*C*) protein abundance. Individual values (*left*) and geometric mean ± 95% confidence intervals (*right*) are displayed on each graph for CON (closed symbols, $n = 7$) and COLD (open symbols, $n = 5$). Each symbol represents one participant (*left*) and is the same for protein and mRNA data (Fig. 6). The horizontal dotted line represents the geometric mean expression at Pre in CON. Muscle was sampled at rest before (Pre) and after 6 wk of training (Post). *$P < 0.05$, different from Pre within group; #$P < 0.05$, different from type I fibers based on pooled group data from both time points (note the line under # for pooled group data).

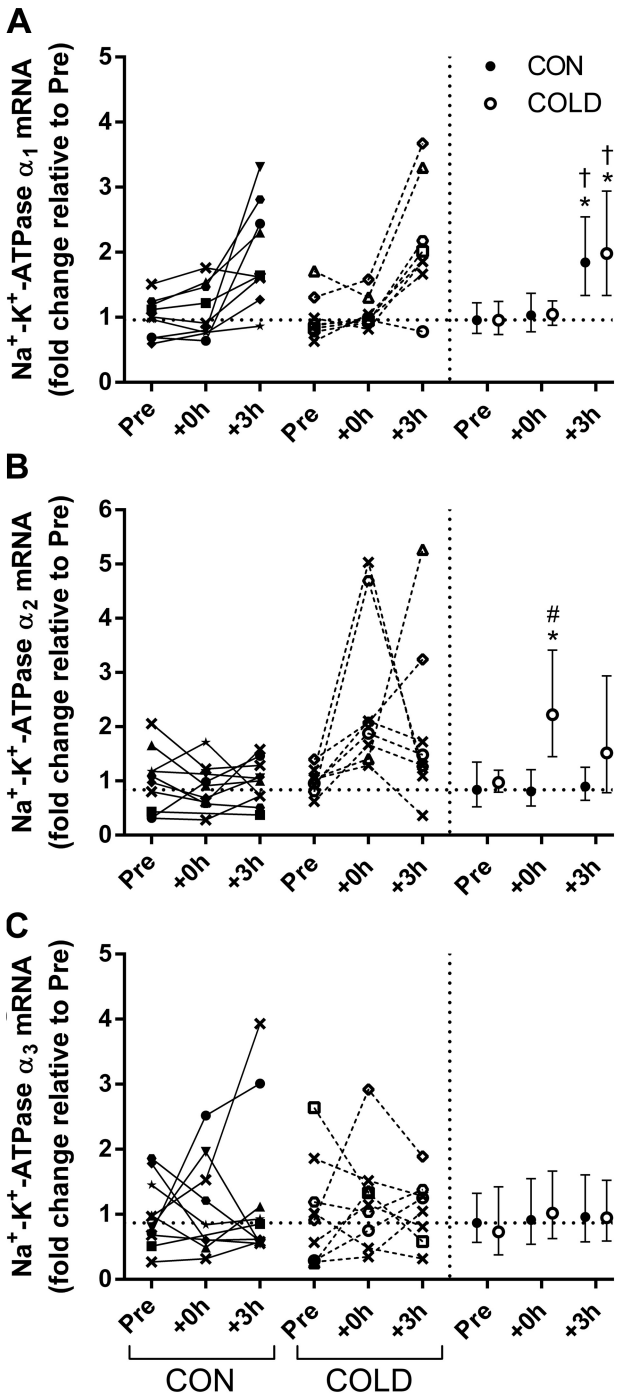


Fig. 6. Effect of a single sprint-interval exercise session with (COLD) or without (CON) cold-water immersion recovery on Na^+,K^+-ATPase α-isoform mRNA content. α_1 (*A*), α_2 (*B*), and α_3 (*C*) mRNA expression. Muscle was sampled at rest before exercise (Pre), 2 min post (+0 h) and 3 h after (+3 h) the assigned 15-min post-exercise recovery treatment. The recovery treatment commenced 4 min after the end of exercise. Individual values (*left*) and geometric mean ± 95% confidence intervals (*right*) are displayed on each graph for CON (closed symbols) and COLD (open symbols). Each symbol represents one participant (*left*) and is the same for mRNA and protein data (Fig. 5). x represents participants from which no muscle sample was left to determine protein abundance. The horizontal dotted line represents the geometric mean expression at Pre in CON. $*P < 0.05$, different from Pre within group; $\dagger P < 0.05$, different from +0 h within group; $\#P < 0.05$, Pre to +0 h change and time point different from CON. In CON, $n = 9$ for α_1, and $n = 10$ for α_2 and α_3. In COLD, $n = 8$ for α_1 and α_2, and $n = 9$ for α_3.

from this session caused a rapid increase in α_2 mRNA content. A visual summary of the main findings is shown in Fig. 9.

Effects of CWI on adaptations in Na^+,K^+-ATPase-isoform content. In contrast to our hypothesis, CWI did not influence fiber type-specific adaptations in muscle Na^+,K^+-ATPase isoforms to sprint-interval training in humans. While these results do not support the use of CWI in an attempt to augment training-induced gains in Na^+,K^+-ATPase content, this provides evidence that CWI can be performed after each training session without impeding protein adaptations of importance for muscle K^+ regulation to intense training. Accordingly, increases (~6%) in performance during incremental, exhaustive cycling and $\dot{V}O_{2max}$ (8%–10%) with sprint-interval training were unaffected by CWI treatment after each training session in the same subjects (7). While enhancements in mitochondrial adaptations to aerobic training have been observed after implementation of CWI in recovery from each training session (27), CWI recovery did not affect increases in mitochondrial content in response to sprint-interval training (7). Thus, effects of regular use of CWI are more apparent after aerobic than anaerobic training, which could at least partly explain the lack of an effect of CWI in the present study. In another study, CWI recovery reduced increases in muscle strength and hypertrophy after resistance training (62). Thus, effects of CWI on training adaptation do also seem to be dependent on the component of muscle function examined, which may be explained by the selective activation of molecular signaling pathways by COLD (45).

CWI invoked an increase (2.2-fold) in α_2 mRNA content after the first training session, which was not evident after the passive recovery treatment. This effect of CWI may have been caused by increased ROS production (64) and/or nonshivering thermogenesis (4), both of which may be potent stimuli to increase α_2 mRNA content in human muscle (47, 55). In contrast, training with CWI did not affect α_2 protein abundance. Thus, CWI may have been an insufficient stimulus to alter effects of training on muscle α_2 protein abundance in the present study. However, the low sample size in COLD for protein measurements ($n = 5$) could have been important for this outcome. In addition, the sprint-interval session alone did not affect α_2 mRNA, which may be explained by the low exercise duration, as other studies in humans reported elevated muscle α_2 mRNA in both untrained (49, 54) and endurance-trained subjects (3) after performing a greater amount of work (2.4- to 3.5-fold).

Reliability of Western blotting for Na^+,K^+-ATPase isoforms. In many human studies, inferences about effects of training on muscle K^+ regulation were based in part or fully on marginal changes in protein abundance (9% to 39%) quantified using Western blotting (21, 23, 26, 52, 67). This may seem surprising considering these studies were limited by use of fiber-type heterogeneous, fractionated samples (51) and, for some, no consideration of blot linearity (44) or antibody lot validation. With use of stain-free imaging technology, wet transfer, and highly sensitive chemiluminescence, we report that in our hands, the technical error of Western blotting using protein from a fiber segment of ~1 to 3 mm in length (~15 μg wet wt tissue), is of a similar proportion (~10% to 30%) as some of the reported changes. Our results highlight that taking method reliability into account in evaluation of changes in abundance

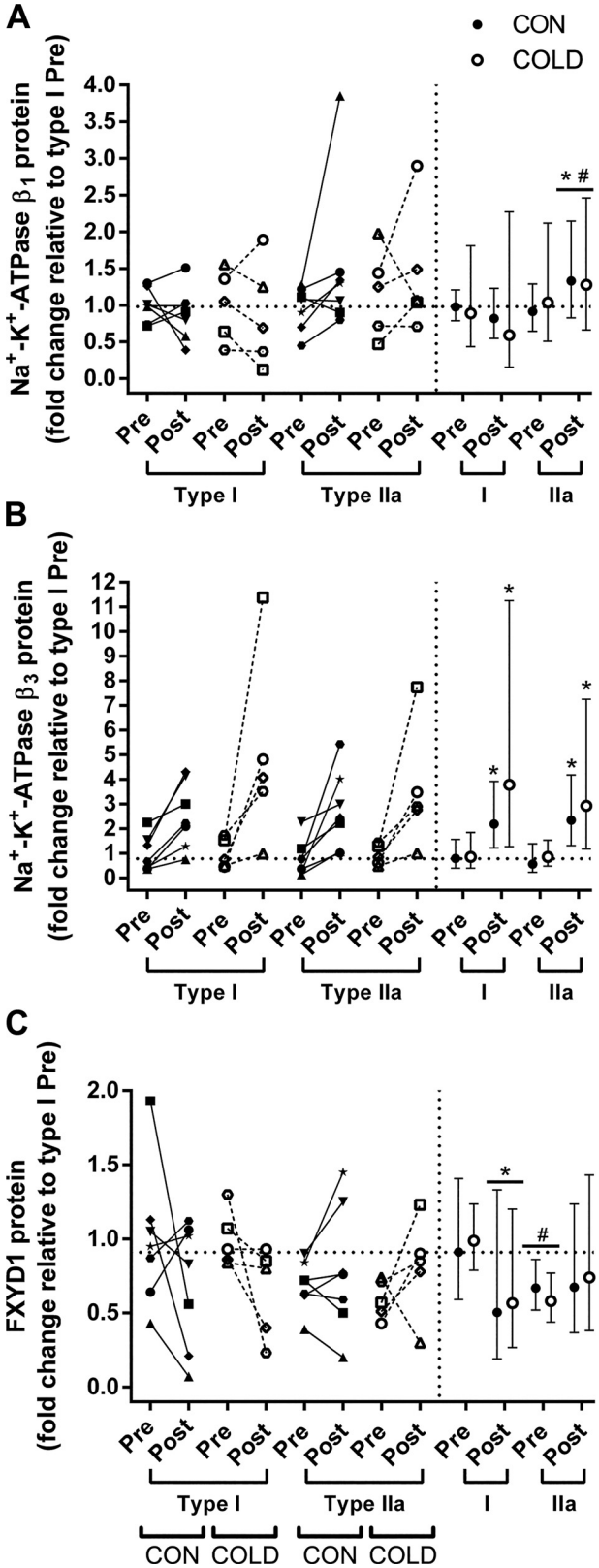


Fig. 7. Effect of six weeks of sprint-interval training with (COLD) or without (CON) cold-water immersion recovery on Na^+,K^+-ATPase β-isoform and phospholemman (FXYD1) abundance in type I and II muscle fibers in men. β_1 (*A*), β_3 (*B*), and FXYD1 (*C*) protein abundance. Individual values (*left*) and geometric mean ± 95% confidence intervals (*right*) on each graph for CON (closed symbols, $n = 7$) and COLD (open symbols, $n = 5$). Each symbol represents one participant (*left*) and is the same for protein and mRNA data (Fig. 8). The horizontal dotted line represents the geometric mean expression at Pre in CON. Muscle was sampled at rest before (Pre) and after 6 wk of training (Post). Note the different scale on the secondary axis and that β_2 protein data were excluded from analysis. *$P < 0.05$, different from Pre within group; $\underline{*}P < 0.05$, different from pooled group data at Pre; #$P < 0.05$, fiber-type difference within time point; $\underline{\#}P < 0.05$, fiber-type difference within time point for pooled group data (note the line under # and * for pooled group data).

of Na^+,K^+-ATPase isoforms is necessary to avoid misinterpretation.

Effects of sprint-interval training on α-isoform abundance in type I and II fibers. Six weeks of sprint-interval training increased α_1 abundance in both type I and II fibers (>2-fold), which contrasts a previous study where sprint-interval training had no effect on α-isoform (α_1, α_2, and α_3) abundance in these fiber types (75). However, both the duration of sprints (4 s) and training (4 wk) may have been too short in the latter study to invoke changes in Na^+,K^+-ATPase α-isoform abundance because other studies in humans found increased [^{3}H]-ouabain binding-site content, reflecting higher α-isoform abundance in whole-muscle samples after sprint-interval training with substantially greater training volumes (24, 42). While lack of sufficient muscle precluded measurement of [^{3}H]-ouabain binding-site content in the present study, our results suggest that increases in [^{3}H]-ouabain binding with sprint-interval training in humans may be explained in part by increased α_1 abundance. Increases in α_1 abundance were certainly clear, with large effect sizes for results in both type I (1.2; $P = 0.008$) and type II (1.6; $P = 0.002$) fibers. Because of the housekeeping role of α_1 in maintaining resting transmembrane Na^+ and K^+ gradients (58), these results strongly suggest that postexercise restoration of Na^+ and K^+ homeostasis may be the major functional outcome of adaptations in α isoforms to sprint-interval training in humans. The lack of significant increase in α_2, whose primary role is to respond to exercise-induced elevations in ion exchange demands (58), supports this suggestion. However, α_2 abundance was quantitatively higher in around three quarters of individual type I and II fiber pools after training. Thus, an increase in α_2 abundance may not have been detected due to interindividual variability and must be regarded. In addition, the rise in α_1 abundance with training was associated with an increase in α_1 mRNA after the first training session, which is within the range of increases previously detected (2.5- to 3.8-fold) in both untrained and trained men after intense intermittent cycling (3, 55) or continuous, fatiguing knee-extensor exercise (49).

Consistent with the lack of effect of training on α_3 abundance, a previous study found no effect of intense intermittent training on α_3 abundance in muscle of well-trained men (3). In agreement with the lack of change in protein with training, no effect of the first training session was observed for α_3 mRNA content. In contrast, high-intensity exercise of substantially longer duration relative to our protocol (40–55 min vs. 2 min) elevated α_3 mRNA content (2.2- to 5.0-fold) in muscle of

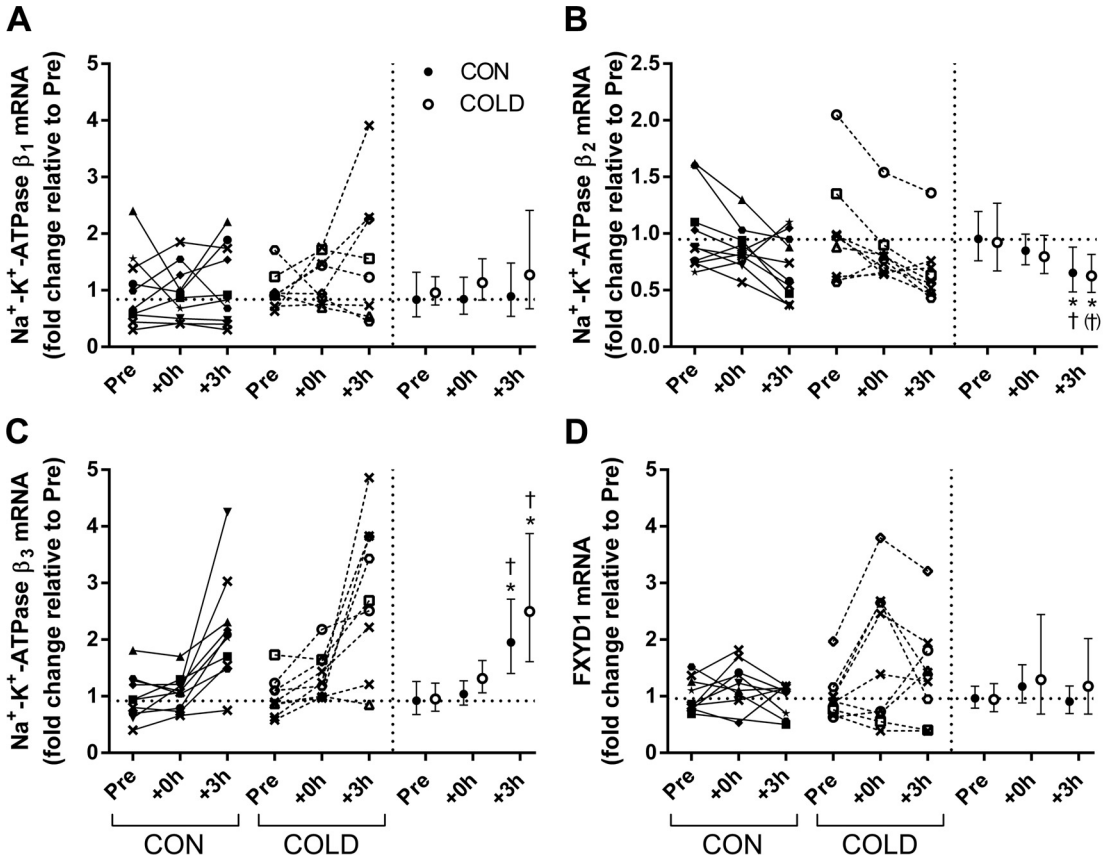


Fig. 8. Effect of a single sprint-interval exercise session with (COLD) or without (CON) cold-water immersion recovery on Na^+,K^+-ATPase β-isoform and phospholemman (FXYD1) mRNA content. β_1 (*A*), β_2 (*B*), β_3 (*C*), and FXYD1 (*D*) mRNA expression. Muscle was sampled at rest before exercise (Pre), 2 min post (+0 h), and 3 h after (+3 h) the assigned postexercise recovery treatment. The recovery treatment was employed from 4 to 19 min after the end of exercise. Individual values (*left*) and geometric mean ± 95% confidence intervals (*right*) are displayed on each graph for CON (closed symbols) and COLD (open symbols). Each symbol represents one participant (*left*) and is the same for mRNA and protein data (Fig. 7). x represents participants from which no muscle sample was left to determine protein abundance. The horizontal dotted line represents the geometric mean expression at Pre in CON. *$P < 0.05$, different from Pre within group; †$P < 0.05$, different from +0 h within group; (†)$P = 0.064$, different from +0 h within group. In CON, $n = 10$ for all genes represented. In COLD, $n = 8$ for β_1, and $n = 9$ for the remaining genes.

untrained (47, 49) and trained men (3), suggesting a large exercise volume may be required to accumulate α_3 mRNA transcripts in human muscle.

Effects of sprint-interval training on FXYD1 abundance in type I and II fibers. Previous studies in humans reported no alterations in FXYD1 abundance in whole-muscle samples after 10 days to 8 wk of intense training (6, 57, 65, 67). In contrast, in the present study, six weeks of sprint-interval training decreased FXYD1 abundance by 33% in type I fibers, whereas no effect was seen in type II fibers. This suggests that an absence of effect of training on FXYD1 abundance in previous studies may be explained by use of fiber-type heterogeneous samples for protein analysis. Sample fractionation (i.e., removal of an indefinite amount of protein) may also have influenced these previous outcomes (51). While lack of blood sampling precluded functional measurements of K^+ homeostasis in the present study, the decline in FXYD1 abundance in type I fibers could have affected K^+ regulation because colocalization of FXYD1 with α/β heterodimers inhibits their activation by increasing K_m (i.e., decreased affinity) for Na^+ and K^+ (15), and interaction of FXYD1 with α_1 or α_2 isoforms remains unaffected by exercise (6).

The first training session was without effect on FXYD1 mRNA content. This finding extends observations in rat muscles of unaltered FXYD1 mRNA after intense exercise (59). In contrast, FXYD1 mRNA was elevated in some subjects when CWI was performed in recovery from the first training session in the present study. This may possibly relate to the stimulating effects of cold on oxidative stress (64), which has been associated with promoted muscle FXYD1 mRNA levels in humans (11). The high interindividual FXYD1 mRNA responses to CWI suggests there may be responders and nonresponders to postexercise CWI.

Effects of sprint-interval training on β-isoform abundance in type I and II fibers. Sprint-interval training increased β_1 abundance (44%) in type II fibers in the present study. This is in agreement with higher type-II fiber β_1 abundance identified in individual fiber segments after short-term sprint-interval training in a mixed-sex human cohort (75). The concomitant increases in β_1 and α_1 abundance in type II fibers in the present study suggest that sprint-interval exercise demands a high K^+ transport capacity of this fiber type.

The first training session did not affect muscle β_1 mRNA content. This is in agreement with absence of effect of different types of exercise on β_1 mRNA content in human muscle (3, 48, 53, 55). In contrast, other studies in healthy men reported elevated (2.4- to 2.8-fold) β_1 mRNA after continuous, moderate-intensity cycling (47) or isolated knee-extensor exercise

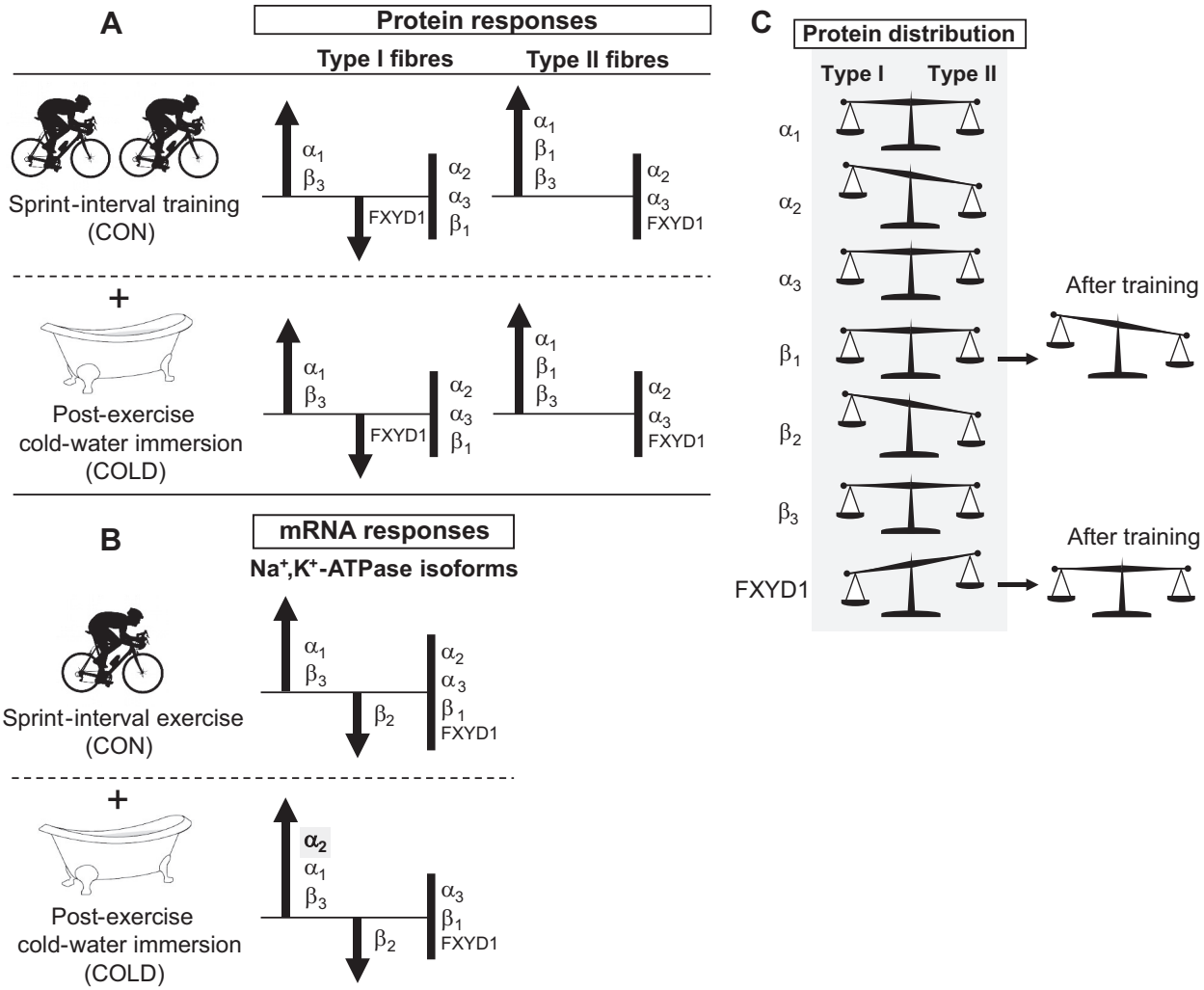


Fig. 9. Summary of key findings. *A*: effect of six weeks of sprint-interval training without (CON) or with 15 min of cold-water immersion (10°C, COLD) after each training session on the protein abundance of Na^+,K^+-ATPase isoforms (α_{1-3} and β_{1-3}) and phospholemman (FXYD1) in type I and II human skeletal muscle fibers. *B*: effect of a single session of sprint-interval exercise without (CON) or with session followed by 15 min of cold-water immersion (10°C, COLD) on the mRNA content of Na^+,K^+-ATPase isoforms (α_{1-3} and β_{1-3}) and FXYD1. Note the selective increase in α_2 mRNA content with COLD. Bold vertical lines without arrow indicate Na^+,K^+-ATPase isoforms that remained unchanged with the given intervention. *C*: protein abundance of Na^+,K^+-ATPase isoforms (α_{1-3} and β_{1-3}) and FXYD1 in type I and II human skeletal muscle fibers, and the effect of the sprint-interval training period on these abundances (after training).

(54). The conflicting outcomes may relate to differences between studies in exercise duration, type, and/or time point of mRNA measurement. For example, our mRNA measurement time point may have reflected a transient state of mRNA with little or no effect on long-term mRNA transcript accumulation, translation, and/or protein synthesis (20).

Despite a lack of assessment of effects of training on β_2 protein, higher β_2 abundance was confidently demonstrated in type II compared with type I fibers, which is in agreement with a previous finding of higher β_2 content in type II muscle fibers after sprint-interval training in humans (75). We also found decreased β_2 mRNA content after the first training session, consistent with a decrease (to ~0.4-fold of Pre level) in the number of β_2 mRNA transcripts in rat EDL muscle after electrical stimulations in vivo (55). In contrast, β_2 mRNA increased (47, 54, 55) or remained unaltered (3, 48, 49, 53) in other human studies. These conflicting findings may partly be explained by different exercise modes used in these studies because sprint-interval and aerobic training induce opposite changes in β_2 mRNA content in rat muscles (59).

The first training session invoked an increase (2-fold) in β_3 mRNA content in the present study. Similar inductions in β_3 mRNA (1.9- to 3.1-fold) have been detected in recreationally active men after knee-extensor exercise (5×2–5 min at 56 W, 3 min rest) (54) and continuous cycling (~75 min at 60%–85% $\dot{V}O_{2max}$) (38). However, changes in β_3 mRNA are not evident in all studies in humans (3, 47–49), which may be explained by highly-trained ($\dot{V}O_{2max}$ ~62–66 $ml \cdot kg^{-1} \cdot min^{-1}$) and sex-heterogeneous cohorts utilized in some studies (48, 49), as these factors are important for increases in β_3 mRNA content in human muscle (46), and by an early time point of muscle sampling after exercise (+0 h) in other studies (3, 47). Furthermore, in the present study, sprint-interval training caused a 2-fold increase in β_3 protein abundance in both fiber types, suggesting that upregulation of β_3 protein could also be relevant for improvements in muscle ion transport function with intense training in humans.

Limitations in measurement of mRNA responses. There are several limitations in methodology for mRNA analysis that should be considered. First, uncertainty of temporal patterns of mRNA decay and synthesis at sampling time points (i.e., fixed in recovery from the first training session) may have been decisive for the present mRNA outcomes given the transient nature and different time courses of increases in mRNA content of Na^+,K^+-ATPase isoforms. Second, use of whole-muscle samples for mRNA analyses may have reduced the validity of the present mRNA measurements because these samples include an indefinite number of type I and II fibers, and Na^+,K^+-ATPase isoforms appear to be expressed in a fiber type-dependent manner in human skeletal muscle (69, 75). In agreement, for isoforms that responded to training in a fiber type-dependent fashion (β_1 and FXYD1), mRNA content determined in whole-muscle samples was unaltered after the first training session. In contrast, upregulation of α_1 and β_3 protein abundance with training, which occurred in both fiber types, was associated with increases in mRNA content of a similar proportion after the first training session. Future research should thus examine mRNA and protein responses in both muscle fiber types simultaneously to exercise sessions over a number of weeks to more accurately determine to what extent transcriptional and posttranscriptional processes participate in fiber type-specific regulation of muscle Na^+,K^+-ATPase-isoform protein abundance by training in humans.

Summary. In summary, six weeks of sprint-interval training invoked fiber type-specific adaptations in the abundance of Na^+,K^+-ATPase isoforms in skeletal muscle of men. For some (α_1 and β_3), but not all (β_1 and FXYD1) isoforms, effects of training on protein abundance were associated with increased mRNA content after the first training session. Furthermore, CWI in recovery from each training session did not interfere with training-induced adaptations in Na^+,K^+-ATPase-isoform abundance in type I and II muscle fibers. Thus, CWI can be performed on a regular basis after training without impairing protein adaptations of importance for muscle K^+ regulation in humans.

ACKNOWLEDGMENTS

We thank Dr. Jujiao Kuang for excellent technical assistance with RT-PCR and RNA extraction. We also thank our participants for kindly donating parts of their muscle and for their efforts throughout the study. The monoclonal antibodies used to measure myosin heavy chain isoforms and the Na^+,K^+-ATPase α_1 isoform in the present study were obtained from the Development Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. The antibodies directed against adult human MHC I and IIa isoforms (A4.840 and A4.74, respectively) were developed by Dr. H. Blau, and the antibody directed against Na^+,K^+-ATPase α_1 (a6F) was developed by Dr. D.M. Fambrough.

GRANTS

This study was partly funded by Exercise and Sports Science Australia (no. ASSRG2011). D. Christiansen was supported by an International Postgraduate Research Scholarship from Victoria University, Melbourne, VIC, Australia.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.C., D.J.B., and J.R.B. conceived and designed research; D.C. and J.R.B. performed experiments; D.C. analyzed data; D.C., D.J.B., J.R.B., M.J.M., and R.M.M. interpreted results of experiments; D.C. prepared figures; D.C. drafted manuscript; D.C., D.J.B., J.B., M.J.M., and R.M.M. edited and revised manuscript; D.C., D.J.B., J.R.B., J.B., M.J.M., and R.M.M. approved final version of manuscript.

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Paper IV

Christiansen D, MacInnis MJ, Zacharewicz E, Xu H, Frankish BP, Murphy RM (2019): A fast, reliable and sample-sparing method to identify fibre types of single muscle fibres. Accepted, Scientific Reports, March 8. Impact factor = 4.609 (5-year).

Title:

A fast, reliable and sample-sparing method to identify fibre types of single muscle fibres

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Short title:

Dot blotting for muscle fibre type identification

Abstract

Many skeletal muscle proteins are present in a cell-specific or fibre-type dependent manner. Stimuli such as exercise, aging, and disease have been reported to result in fibre-specific responses in protein abundances. Thus, fibre-type-specific determination of the content of specific proteins provides enhanced mechanistic understanding of muscle physiology and biochemistry compared with typically performed whole-muscle homogenate analyses. This analysis, however, is laborious and typically not performed. We present a novel dot blotting method for easy and rapid determination of skeletal muscle fibre type based on myosin heavy chain (MHC) isoform presence. Requiring only small amounts of starting muscle tissue (*i.e.*, 2-10 mg wet weight), muscle fibre type is determined in one-tenth of a 1-3-mm fibre segment, with the remainder of each segment pooled with fibre segments of the same type (I or II) for subsequent protein quantification by western blotting. This method, which we validated using standard western blotting, is much simpler and cheaper than previous methods and is adaptable for laboratories routinely performing biochemical analyses. Use of dot blotting for fibre typing will facilitate investigations of fibre-specific responses to diverse stimuli, which will advance our understanding of skeletal muscle physiology and biochemistry.

Keywords: Single fibres; fibre type; dot blotting; Western blotting; muscle physiology; muscle biochemistry

Introduction

Skeletal muscle is a heterogeneous and dynamic tissue with respect to fibre type. In humans, skeletal muscle fibres are characterised according to the presence of myosin heavy chain (MHC) isoforms as type I, IIa, and IIx, but I/IIa and IIa/IIx hybrid fibres are also present [1-5]. Fibre types have different biochemical and physiological properties. In comparison to type II ("fast twitch") fibres, type I ("slow-twitch") fibres have a slower rate of force production and sarcoplasmic reticulum (SR) Ca^{2+} kinetics, altered glycogen utilization, possess more mitochondria, and are more resistant to fatigue [6-12]. Reflecting their distinct physiology and biochemistry, protein content and metabolic regulation differ across skeletal muscle fibre types. In large muscles, fibres are recruited according to the force output required (type I before type II), and the proportion of each skeletal muscle fibre type is associated with the function of a particular muscle and the characteristics of the individual (*e.g.,* genetics, fitness, and age) [13]. Thus, fibre type-specific protein analyses are critical for a more mechanistic understanding of skeletal muscle physiology.

The many published studies that have addressed fibre-specific aspects of skeletal muscle physiology, metabolism, and biochemistry required tedious, costly, and time-consuming sample collection and analyses [6-11,14]. Previously, our research group presented a method for analysing protein content in fibre segments (~1-3-mm segments) of freeze-dried skeletal muscle fibres [15]. Using this method, which is predicated on sensitive Western blotting, it is possible to simultaneously determine the fibre type and relative abundance of proteins of interest in an individual fibre segment. This method, or modifications of it, has been used successfully to study skeletal muscle cell physiology at the fibre type level [16-18] as well as skeletal muscle fibre-specific responses to acute [19-21] and chronic exercise [22,23], aging [17,24], and disease [25]. Whilst this method drastically reduced the time required for fibre type-specific analysis compared to earlier investigations [6-11], it remains somewhat hindered by the time and costs associated with separately analysing protein content in 100s to 1000s of individual muscle fibre samples for a single study.

In this manuscript, we describe an advance in the analysis of fibre-type specific protein content in skeletal muscle biopsy samples. Briefly, fibre segments were collected, and

the equivalent of ~1/10 of each fibre segment was dot blotted for detection of MHC IIa and proteins. According to this detection, fibre segments were pooled to form samples of type I and type IIa fibre segments for Western blot analysis [20,25]. The main advantages of this method are that it requires very little sample (*i.e.,* ~2-10 mg wet-weight tissue is sufficient for single fibre analyses, compared to 20-60 mg used previously [15,19,32]), is simple and quick to perform and drastically reduces the costs (~40-fold) associated with fibre type-specific muscle analyses. While the presented method is advantageous in detecting type I and IIa fibres (and IIx fibres through a process of elimination using antibodies specific to other MHC isoforms) compared to existing methods, it cannot be used to detect hybrid IIa/IIx fibres at present due to cross-reactivity of the IIx antibody with other MHC isoforms.

Results

Comparison of dot blotting and Western blotting for fibre type determination

The fibre types of the fibre segments were identified by dot blotting the equivalent of 1/10 of each fibre segment sample (i.e. 1 μL) to PVDF membrane and sequentially probing with antibodies against MHC IIa, MHC I, and MHC IIx. The first probe identified 13 of the 20 fibre segments as being positive for MHC IIa, with the two whole-muscle control samples also positive for MHC IIa (Fig. 2A, top). Those fibre segments that did not contain MHC IIa have been indicated with a circle, and following the second probe with MHC I, two of those fibre segments were positive (Fig. 2A, middle, red circles showing the MHC I positive fibres). The final probe showed that most fibre segments were immunoreactive with MHC IIx antibody (Fig. 2A, bottom). Thus, this antibody is clearly unspecific when used in dot blotting (i.e., it detects multiple MHC isoforms). It is clear, however, that four of the blue-circled fibre segments had not stained previously for MHC IIa or MHC I, but now stained positive for the first time using the MHC IIx antibody and these fibre segments were therefore identified as type IIx fibres, through a process of elimination (blue circles, Fig. 2A). Hybrid I/IIa fibre segments can clearly be detected, as seen in a further subset of fibre segments which reacted with MHC IIa and/or MHC I antibodies, identified with blue (MHC IIa), red (MHC I) and purple (MHC I/IIa) circles (Fig. 3).

To validate the dot-blotting results, ⅛ of each of the same samples were run on Criterion Stain Free SDS-PAGE gels. Following transfer to membrane, the membrane was probed with the same MHC antibodies as used for dot blotting. In all cases, fibre segments identified as type I, IIa or IIx using dot blotting (Fig. 2A) showed the same identification using Western blotting (Fig. 2B). The fibre segment in position B10 was not identified with dot blotting, but with Western blotting was revealed to be a type IIx fibre segment. The lack of identification using dot blotting was likely due to the small size of that fibre segment, which was confirmed by the Stain Free gel image of fibre total protein content (*e.g.,* compare lanes of fibre segments B9 and B10 that demonstrate the much lower total protein and hence fibre segment size of B10). Thus, the concordance between the two techniques was 100%.

Fibre type dependence of protein abundance

Following fibre type verification by Western blotting, the membranes were probed for SR Ca^{2+}-ATPase isoforms 1 (SERCA1) and 2 (SERCA2a), the calcium-binding protein, calsequestrin, isoforms 1 (CSQ1) and 2 (CSQ2), the contractile protein, Actin and the beta 2 subunit of the energy-sensing protein, AMP kinase (AMPKβ2) (Fig. 4 and S1). SERCA1 and SERCA2a were only present in type II or type I fibres, respectively. CSQ content was variable between the fibre types, although CSQ1, the fast (type II) isoform, was least abundant in type I fibres, which showed the highest expression of CSQ2, the slow (type I) isoform. These provide some validation that the MHC antibodies are specific for fast and slow-isoforms, as previously shown [26,27]. Actin was similarly abundant in fibres, showing a similar expression to that of the total protein gel, as expected for a contractile protein that is abundant in both fast and slow-twitch muscle fibres. The expression of AMPKβ2 showed the most variability across the fibres, regardless of the MHC isoforms present (Fig. 4).

Variation and reliability of Western blotting for individual skeletal muscle fibres

We sought to quantify the variability in total protein content, as well as Western blot signal and normalised content of proteins of interest across individual type I and IIa skeletal muscle fibres collected from a single human muscle biopsy, to ensure only intra-individual biological variability would be present. As shown by the CV and IQR in Supplementary Table S1 online, there was considerable variability in the total protein

content of type I and IIa fibres (i.e., the amount of protein in each 4-µL aliquot) and the Western blot signals for the proteins of interest. This was as expected, because fibre size was purposely not heavily considered when fibres were being isolated, simply because fibre size is weighted heavily on fibre radius (r, typically 30-40 µm) and less on fibre length (l, typically 1-3 mm, where volume = πr^2 l) and radius (as seen by the fibre diameter) cannot be accurately assessed using a dissecting microscope. This aside, ICC values were good to excellent, and typical errors (as a CV) were mostly below 20%, suggesting that these measures were reliable. Further, the subsequent analyses accounted for variability in fibre size, whereby normalising the Western blot signals to the total protein content (Supplementary Table S1 online) decreased the CVs and IQR for normalised protein contents considerably, owing to the moderate to strong linear relationships between total protein content and Western blot signal (Figure 5). While the ICC values were lower for normalised protein content than raw values (Table 1), they were still moderate to good, and the typical errors (as a CV, Table 1) were mostly unchanged, demonstrating good reliability of the technique.

Validation of pooling muscle fibres

Given the variability of normalised protein content in fibres of the same type (Supplementary Table S1 online), we proceeded to investigate the theoretical variability associated with the pooling of different numbers of fibres from the same skeletal muscle biopsy. We performed this analysis through a simulation that executed each sampling protocol (i.e., sampling 1 to 40 fibres) 1000 times, using the datasets generated from the 40 type I and type IIa fibres. As shown in Table 2, the width of the 95% confidence intervals associated with pooling different numbers of type I and IIa fibres decreased with increasing numbers of pooled fibres. Breakpoint values ranged from 3 to 9 fibres, after which all slopes were relatively small (i.e., <0.03 a.u. reduction in the 95% confidence interval width per additional fibre added to the pooled sample).

Discussion

This manuscript presents an advance in methodology for fibre type identification of skeletal muscle fibres that can easily be introduced into laboratories studying skeletal muscle physiology and biochemistry. The work expands previous progress made in the ability to analyse proteins in segments of single skeletal muscle fibres [15]. By following our prescribed methodology, the ability to collect and analyse skeletal muscle samples for relative, quantitative measurements of proteins in specific fibre types is very accessible. This new method saves considerable time and money, because no gels or transfer step are needed and a less sensitive chemiluminescence reagent can be used. We estimate that this method reduces the cost to fibre type 50 fibre segment by ~40 fold (AUD $64 vs $1.40).

Our first key finding is that qualitative determination of fibre type of an isolated muscle fibre can be performed using dot blotting of $\frac{1}{10}$ of a 1-3 mm fibre segment dissected from a human muscle biopsy. The validity of dot blotting was demonstrated by Western blotting of another portion of the same fibre segment and detecting MHC isoforms (Fig. 2), as performed frequently by us [15-19,22,24,28] and others [20,25]. Thus, whilst either technique could be used to determine the fibre type of individual muscle fibre segments, the dot blotting procedure is much quicker and simpler to perform. It allows 100s of individual fibres to be fibre typed in one day in contrast to 10s of fibres over two or more days using conventional Western blotting, Furthermore, dot blotting is dramatically cheaper because no gels or transfer steps are required and a less sensitive chemiluminescence reagent can be used. Previously, protein densitometry analyses had to be performed separately for each fibre segment before averaging the data, which markedly increased the time required for fibre-specific analysis. With dot blotting, rapid detection of fibre type proceeds pooling of fibres, and proteins can then be quantified at the pooled-fibre level using modified Western blotting [20,21,25]. While there might be potential in the presented method to determine an individual's fibre-type distribution of a given muscle biopsied, this would require a much larger number of fibre segments be dissected and that there be no bias in selection of fibres from the muscle biopsy. Thus, in its presented form, we do not recommend dot blotting for assessing fibre-type distribution in a muscle biopsy.

We demonstrate the ability to identify MHC I/IIa hybrid fibres using dot blotting (Fig. 3).

In this regard, when considering if a given fibre is a hybrid fibre, we suggest that having <5% of a given MHC isoform will not greatly alter speed of contraction of a muscle fibre over the predominant MHC present, and consequently, a threshold of <5% could be used as cut-off to determine whether a fibre should be characterized as a hybrid. Further, we were able to identify single fibres expressing only the MHC IIx isoform with the 6H1 (MHC IIx) antibody using Western blotting. In dot blotting, however, this antibody did cross-react with other MHC isoforms. Despite this cross-reactivity, an unequivocal determination can be made for MHC IIx fibres through a process of elimination: although all samples were positive for MHC IIx, we demonstrated, by Western blotting, that those fibres that were negative for MHC I and MHC IIa but positive for MHC IIx were indeed MHC IIx fibres segments (Fig. 2A). We note that it is not possible to detect hybrid type IIa/IIx fibres, due to cross-reactivity with other MHC isoforms of the antibody used to detect MHC IIx. Indeed, sequence identity of MHC I (Myosin-1[Homo sapiens], Accession NP_005954) and IIx (Myosin Heavy Chain IIX/D [Homo sapiens], Accession AAD29951) revealed only a single amino acid difference between the two proteins (Protein Blast analyses, PubMed). With IIa/IIx fibres possibly accounting for 3-10% of the total skeletal muscle fibre pool [29], and considering the relative proportion of these hybrid fibres within the total fibre pool may increase with certain disease states or in muscle obtained from old individuals, the cross-reactivity of this antibody is currently a limitation of the presented method.

To clarify the number of fibres required for valid quantification of fibre type-specific protein content, we addressed three potential issues: (i) the variability in protein content in type I and type IIa skeletal muscle fibres from a single human muscle biopsy; (ii) the relationships between Western blot signals and total protein content; and (iii) the variability associated with different numbers of pooled fibres. As we have previously highlighted [30], the variability of normalised protein content within a given fibre type from a given individual is not trivial (CVs between ~15-25% in the present experiment). This variation was much lower than the variation in total protein content and Western blot signal (generally > 50%), which, as stated above, was as expected because fibres of different lengths and diameters were indiscriminately sampled. Due to

the moderate to strong linear relationships between total protein content and Western blot signal, using calibration curves and normalising to total protein content accounted for these differences markedly. This finding means there is no issue in loading different amounts of protein (i.e., different size fibres) and obtaining accurate results. Further, this outcome appears independent of protein size, as similar ICC values were obtained for all the investigated proteins, despite large differences in protein size (e.g., Figure 4). Note that ICC values were lower for normalised protein content than for total protein content or the Western blot signal (Table 1), but this finding is due to the reduction in variance that occurs with normalisation [31]. Typical errors (as a CV) were unaffected by normalisation and remained below 20%.

Finally, we demonstrate that breakpoints in the 95% confidence interval widths occurred between 3 and 9 fibres. While the breakpoint does not necessarily indicate the correct number of fibres to pool, diminishing returns with respect to the accuracy of the pooled fibre mean were apparent when additional fibres were added to the pool. These number of fibres are typical of what has been used in the literature [21,23,32]. Further, other defining factors for the number of fibres to be pooled will also be the number of target proteins and the anticipated statistical power. We have demonstrated the ability to measure specific proteins in a typical muscle fibre segment, and also that in order to be quantitative it is most useful to use small sample sizes [33]. As such, the amount of sample corresponding to a pool of 4 fibres (i.e., ~36 µL after dot blotting 1 µL per fibre) will be sufficient in volume to run 4 gels and subsequent western blots, but if there is a greater number of proteins of interest, requiring more gels to be run, then pooling more fibres to provide sufficient sample should be considered during the experimental design of the western blot analyses. Theoretically, adding more than 3-9 fibres to a pooled sample may be necessary for some study designs to accurately represent a total muscle fibre pool. For example, if statistical power is low (e.g., a small difference between groups, and a between-subject design is used), then more fibres will be needed per pooled fibre-type sample to reduce experimental noise. In contrast, if statistical power is large (e.g., a large difference is anticipated between groups, and a within-subject design is used), then relatively few fibres will be needed per pooled fibre-type sample. Further, it should be considered that this minimum number of fibres to pool (3-9) is restricted to the n=2 healthy humans assessed in the current study. For example, other

populations, such as those with diabetes or endurance-trained athletes, might introduce heterogeneity with respect to the biochemical and physiological properties of a given fibre type. This may alter the variability in expression of proteins in different fibre types, and hence the minimum number of fibres required for reproducible protein analysis.

In summary, in skeletal muscle, many proteins involved in a variety of different cellular functions are expressed in a fibre-specific manner. Also, fibre type-specific protein responses to interventions such as a single bout of exercise [19], exercise training [20,22], or processes like disease [25] and aging [24] have been reported. Such findings highlight the advantage of addressing fibre type heterogeneity in skeletal muscle protein analyses, as well as the necessity to do so. Failure to do so implies that physiologically relevant changes may be overlooked [19,22,24]. Somewhat hindered in the past by the need for highly laborious and time-consuming experiments, the exciting advance presented here provides the means for fibre type-specific studies to be undertaken far more easily in most laboratories. The method presented, and our demonstrated ability to reliably measure the abundance of proteins of varying absolute abundance in groups of only a few fibres, opens the way for improvements in our understanding of how muscle fibre type may play a crucial regulatory role in skeletal muscle physiology.

Methods

Ethical approval and muscle biopsy procedure

The skeletal muscle samples (n=2) were collected as part of a study approved by the Hamilton Integrated Research Ethics Board (Hamilton, Canada: Study approval: 15-357). The study conformed to the Declaration of Helsinki II and participants provided informed consent prior to participation. Separate data from this study have been published (MacInnis et al. 2016). Both participants were males who performed structured exercise 2-3 times per week but were not training for any specific sport. Muscle biopsy samples were collected at rest prior to an exercise training intervention. The participants had an average age of 26.0 (4.2) years, a body mass index of 27.4 (3.0) kg/m2, and a maximum oxygen uptake of 49.6 (2.3) mL/kg/min. Briefly, samples were obtained from the *vastus lateralis* muscle under local anaesthesia (1% Xylocaine) using

a Bergström needle with suction. The study volunteers were healthy males, non-smokers, and engaged in physical activity several days per week.

Chemicals

General chemicals were from Sigma (Sydney, Australia) and Western blotting solutions and consumables were from BioRad (Hercules, CA, USA) unless otherwise stated.

Collection of skeletal muscle fibre segments

A muscle sample was freeze-dried for 48 h, brought to room temperature, and 1-3 mm segments of individual muscle fibres were removed under a microscope using jeweler's forceps, as described by Murphy [15] (Fig. 1). The average normalized length of a skeletal muscle fibre in human *m. vastus lateralis* is ~6.6 cm [34], with an optimal fibre length of ~11.1 cm [35]. As such, the 1-3 mm lengths of fibre segments collected from muscle biopsies were a small section of any given muscle fibre. Each fibre segment was added to a 0.6-mL tube containing 10 µL of SDS loading buffer (0.125 M Tris-HCI, 10% glycerol, 4% SDS, 4 M urea, 10% 2-mercaptoethanol and 0.001% bromophenol blue, pH 6.8 diluted 2:1 with 1x Tris-HCl (pH 6.8)). Muscle samples were solubilized by vortexing for 5-10 s and exposing to room temperature for 1-2 h, and were then stored at -80°C until analysis.

Dot blotting procedure

A PVDF membrane was activated in 95% ethanol for 15-60 s and then equilibrated for 2 min in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3 and 20% methanol). The wet membrane was placed on a stack of filter paper (one to two pieces soaked in transfer buffer on top of three dry pieces). Samples were thawed and vortexed, but were not centrifuged to avoid pelleting and hence loss of any of the skeletal muscle protein. Samples were spotted to a specific part of the membrane in aliquots equating to $\frac{1}{10}$ of a fibre segment (i.e., 1 µL) using a pipette. An aliquot of whole-muscle crude homogenate was added in triplicate as positive controls for MHC I and IIa positive fibres. After complete absorption of samples, the membrane was placed on top of a dry piece of filter paper to dry for 2-5 min before being reactivated in 95% ethanol for 15-60 s and equilibrated in transfer buffer for 2 min. After three quick washes in Tris-buffered saline-Tween (TBST), the membrane was blocked in 5% non-fat milk in TBST

(blocking buffer) for 5 min at room temperature. Following blocking, the membrane was rinsed with TBST and then incubated in MHC IIa antibody (mouse monoclonal IgG, clone A4.74, Developmental Studies Hybridoma Bank [DSHB], 1 in 200 in 1% BSA/PBST) at room temperature for 2 h with gentle rocking. Membranes were washed in blocking buffer and then incubated in goat anti-mouse IgG horse radish peroxidase (HRP) secondary antibody (ThermoFisher Scientific: PIE31430, 1 in 20,000 in blocking buffer) at room temperature for 1 h with rocking. Lastly, membranes were washed in TBST and then exposed to Clarity enhanced chemiluminescence reagent (BioRad, Hercules, CA, USA), imaged (ChemiDoc MP, BioRad), and analysed for signal density (ImageLab 5.2.1, BioRad).

The MHC IIa antibody and its secondary antibody were removed from the membrane with stripping buffer (Pierce, Rockford, IL, USA) prior to incubation of the membrane in MHC I antibody (mouse monoclonal IgM, clone A4.840, DSHB, 1 in 200 in 1% BSA/PBST) and goat anti-mouse IgM secondary antibody (Santa Cruz Biotechnology, TX, USA: sc-2064, 1 in 20,000), using the same procedure as for MHC IIa. In some circumstances, a similar process was repeated using the MHC IIx antibody (mouse monoclonal IgM, clone 6H1 DSHB, 1 in 100 in 1%BSA/PBST) and the goat anti-mouse IgM secondary antibody. Using images of all the membranes, it was possible to determine the fibre type of each sample (I, IIa, I/IIa hybrid, IIx) or if no MHC protein was present, which would indicate unsuccessful collection of a fibre segment (Fig. 2). Whilst stripping of membranes may remove a small quantity of protein sample, it is valid in the current setting for MHC I and MHC IIa, because qualitative, and not quantitative, results are required for fibre typing.

<u>Confirmation of the dot blotting procedure</u>

Following the method of Murphy [15], a 2-µL aliquot of each sample that was dot blotted, equating to ~⅛ of a fibre segment, was run on a 4-15% Criterion TGX Stain-Free protein gel (BioRad) at 200 V for 45 min. Total protein on the gel was visualised with UV activation, which involved exposure of the gel to UV light that activates endogenous tryptophan amino acids present in the proteins, and an image similar to a coomassie stained gel captured (StainFree Imager, BioRad). Proteins were then wet-transferred to nitrocellulose membrane at 100 V for 30 min in circulating 4°C transfer buffer. Proper

transfer was visualised with the same UV activation as described above, but images were captured from both the gel and the membrane. Membranes were treated with Miser solution (ThermoFisher Scientific) and placed in blocking buffer for 2 h at room temperature before being subjected to a similar Western blotting protocol as described for the dot blotting procedure, except, other than MHC probes, no stripping of membrane between any of the antibody probes. A fibre segment was considered to not express a given MHC isoform if its abundance was <5% of the density seen in other positive lanes. To demonstrate fibre type-dependent protein abundance, in addition to probing for MHC isoforms in the region of the blot above 170 kDa, typically the lower portions of these membranes were cut into three regions (including markers: 95 and 130 kDa; 40 and 55 kDa; 17, 26 and 35 kDa) probed with antibodies (diluted in 1% BSA/PBST) against SERCA1, (mouse monoclonal, DSHB, CA F2-5D, 1 in 1000) and SERCA2 (Badrilla, A010-2,1 in 5000); CSQ1, (mouse monoclonal, Abcam:ab2824 , 1 in 2000) and CSQ2 (rabbit, Abcam:ab3516 , 1 in 1000); Actin (rabbit polyclonal, Sigma A-2066, 1 in 300); AMPKβ2, (rabbit monoclonal, Cell Signalling #4150, 1 in 1000). For the determination of the linear relationships between the fibre segments containing MHC I or MHC IIa, the mitochondrial content marker, cytochrome c oxidase subunit IV (COX IV, rabbit polyclonal, Cell Signalling #4844, 1 in 1000) was also used. Secondary antibodies were as above or goat anti-rabbit HRP (ThermoFisher Scientific: PIE31460, 1 in 60,000) where suitable. Images of the membrane were captured with the Chemidoc MP after chemiluminescence and then white light images taken without moving the membrane to allow for merging of markers and chemiluminescent imaging.

Validation of fibre pooling and protein analyses

Another muscle biopsy was freeze-dried for 48 h and dissected. Individual fibre segments were collected, and a small fraction ($1/10$) of the samples was used to identify fibre type, using the dot blotting method described above, until 40 type I and 40 type IIa fibre segments were identified. Western blotting was performed on aliquots (4-µL) of these individual fibre segments, as described above. Twenty individual fibre segments of each fibre type, and a calibration curve of mixed muscle homogenate (i.e., 1, 2, 4, and 8 µL), were run in separate lanes on each gel. The total protein content and abundance of SERCA1 (type-IIa specific), SERCA2A (type-I specific), AMPKβ2, and COXIV were determined in each fibre segment sample, as described above, with the membrane cut

to allow for minimal probes of given regions. Calibration curves were used to express abundances on the same scale across gels. Normalised protein abundance was calculated by dividing the Western blot signal of each protein of interest by the total protein content of the sample, which has also been expressed relative to its calibration curve. All samples were run in duplicate on separate gels. Consequently, protein quantification was performed in duplicate for each fibre segment sample.

Statistics

In type I and type IIa fibre segments, n = 40 for all datasets. The mean, standard deviation, 95% confidence interval, coefficient of variation (CV), and interquartile range (IQR) were calculated in Excel (MS Office 2013, Microsoft, USA) for the total protein content, as well as both Western blot signal and normalised protein abundance of SERCA1, SERCA2A, AMPKβ2, and COXIV after averaging duplicate values. Normality was tested using the D'Agostino and Pearson omnibus normality test in GraphPad Prism (Ver. 6, GraphPad Software, USA). Using the spreadsheet provided by Hopkins [36], reliability of the Western blotting protocol was determined by calculating the typical error (in A.U. and as a CV) and intraclass correlation coefficient (ICC) for duplicate measures of the total protein, as well as Western blot signal and normalised abundance of proteins of interest. ICC values < 0.5, between 0.5 and 0.75, between 0.75 and 0.9, and >0.9 were considered to represent poor, moderate, good, and excellent reliability, respectively [37]. To determine the relationships between total protein content and Western blot signal for each protein of interest, linear regressions and coefficients of determination (R^2) were performed in GraphPad Prism. Outliers were removed from the linear regression analyses using the ROUT method, with a Q value of 1% (between 0 to 5 samples per protein; see Fig. 5).

Using the datasets of 40 individual fibre segments to represent a whole skeletal muscle biopsy sample, a fibre pooling simulation was performed *in silico* to establish the accuracy of the mean achieved when different numbers of fibre segments were pooled. Briefly, a script was written in R (r-project.org) that randomly selected (with replacement) a specific number of fibre segments from a given dataset and then calculated the mean normalised protein content of this sample. This procedure was repeated 1000 times for all sample sizes between 1 and 40 fibre segments, and the 95%

confidence interval of the mean associated with each sample size was determined. Using the "segmented" package in R, the breakpoint in the 95% confidence interval width was determined to identify the sample size at which adding additional fibre segments to the pool began to have a relatively smaller impact on the accuracy of the mean. These procedures were performed for each protein of interest in each fibre type.

Data Availability Statement

All authors adhere to the data availability requirement for publication in Scientific Reports and support materials, data and associated protocols are made promptly available to readers without undue qualifications in material transfer agreements.

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Acknowledgements

We thank Ms. Heidy Latchman for technical assistance.

Author contributions

DC, MM and RMM conceived the study; DC, MM, EZ, HX, BPF, DC and RMM designed, performed and analysed the experiments. DC, MM and RMM wrote the manuscript. DC, MM, EZ, HX, BPF and RMM all approved the final version of the manuscript.

Competing Interests Statement

No conflicts to report.

Figure legends

Figure 1. *Representative images of a freeze-dried muscle fibre bundle (A) and a fibre (B) at 20x magnification.* Scale bar as indicated.

Figure 2. *Fibre segments from human vastus lateralis muscle identified for myosin heavy chain (MHC) isoforms by dot blotting and Western blotting*-(**A**) $^{1}/_{10}$ of each fibre segment was dot blotted (positions A1-10, B1-10), along with control muscle samples (+ lane). Based on MHC isoform expression, fibre segments were characterised as type I (red circles), IIx (blue circles), or IIa (no circle). There was one dot/sample that remained negative for all three probes (green circles). (**B**) Fibre type of fibre segments from positions A1-10 and B1-10, was also determined by Western blotting using ⅕ of each fibre segment sample. The 170 kDa molecular marker can be seen for each blot (lanes labelled M). A single Stain Free gel is shown as a representation of the gels, and is indicative of the total protein loaded. Lanes are numbered according to the positions on the dot blots in (A) and below are indicated the assigned fibre type. Thirteen type IIa (black text), two type I (red text), and four type IIx fibre segments (blue text) were positively identified. Fibre type of the fibre segment at position B10 could not be determined using dot blotting, but was characterised as a type IIx fibre using Western blotting (green text).

Figure 3. *Fibre segments identified as hybrid (purple circles) for myosin heavy chain isoforms by dot blotting*. (**A**) Fibre segments were identified as type IIa (circled in blue), type I (circled in red), or as hybrid (I/IIa; circled in purple). Note red dots are present as the membrane was purposely overexposed to maximise the ability to observe dots of weaker intensity. Classification of each fibre analysed shown in (**B**).

Figure 4. *Fibre-type specific expression of various proteins in fibre segments*.
The 14 fibre segments shown in the Western blot in Figure 2B were probed for SERCA1, CSQ1, and AMPK β2 (1st probes in the respective regions of the membrane) and SERCA2a and CSQ2 (2nd probes), and Actin (3rd probe), with no stripping of membranes between probes. As seen, the fibre type determined by dot blotting of each fibre segment (Dot-blot fiber ID) corresponds to the expected fibre-specific expression of these proteins, as determined by western blotting. Note that the CSQ antibody detected both CSQ1 (upper band) and CSQ2 (lower band). As in Fig. 2, the fibre type identification is indicated above the blots and below the Stain Free gel. Sizes of molecular weight markers are indicated on the left of each blot, whereas sizes for the proteins of interest are on the right.

Figure 5. Linear relationships between Western blot signals and total protein content for SERCA2A/SERCA1 (A), AMPKβ2 (B), and COXIV(C) in individual type I (black squares) and type IIa (open circles) human skeletal muscle fibre segments. Sample sizes, equations for linear relationships, and coefficients of determination are shown for each fibre type. The solid line indicates the line of regression, and the dashed lines indicate the 95% confidence interval of each linear regression.

Table 1. Measures of reliability for total protein content, Western blot signals, and normalised protein content for proteins of interest in individual type I and type IIa human skeletal muscle fibre segments.

Fibre type	Protein [a]	Protein content or Western blot signal			Normalised protein content		
		Typical Error, a.u. [b]	Typical Error as CV, %	ICC [c]	Typical Error, a.u. [b]	Typical Error as CV, %	ICC [c]
Type I	Total	0.12 (0.10-0.15)	11.5 (9.3-15.0)	0.97 (0.94-0.98)	-	-	-
Type I	SERCA2A	0.85 (0.70-1.10)	22.4 (18.0-29.7)	0.97 (0.95-0.98)	0.78 (0.64-1.00)	20.3 (16.4-26.8)	0.70 (0.50-0.83)
Type I	AMPKβ2	0.25 (0.21-0.33)	16.2 (13.1-21.3)	0.87 (0.77-0.93)	0.20 (0.16-0.25)	18.2 (14.7-24.0)	0.50 (0.23-0.70)
Type I	COXIV	0.64 (0.52-0.82)	19.5 (15.7-25.7)	0.81 (0.68-0.90)	0.30 (0.25-0.39)	15.3 (12.3-20)	0.67 (0.46-0.81)
Type IIa	Total	0.26 (0.21-0.33)	8.0 (6.5-10.4)	0.96 (0.93-0.98)	-	-	-
Type IIa	SERCA1	0.59 (0.48-0.76)	11.4 (9.3-14.9)	0.90 (0.82-0.94)	0.09 (0.08-0.12)	9.6 (7.8-12.5)	0.87 (0.78-0.93)
Type IIa	AMPKβ2	0.40 (0.33-0.51)	15.4 (12.5-20.2)	0.86 (0.75-0.92)	0.09 (0.08-0.12)	10.7 (8.7-14.0)	0.56 (0.31-0.74)
Type IIa	COXIV	0.83 (0.68-1.06)	16.8 (13.5-22.0)	0.87 (0.77-0.93)	0.13 (0.11-0.17)	11.2 (9.1-14.6)	0.82 (0.68-0.90)

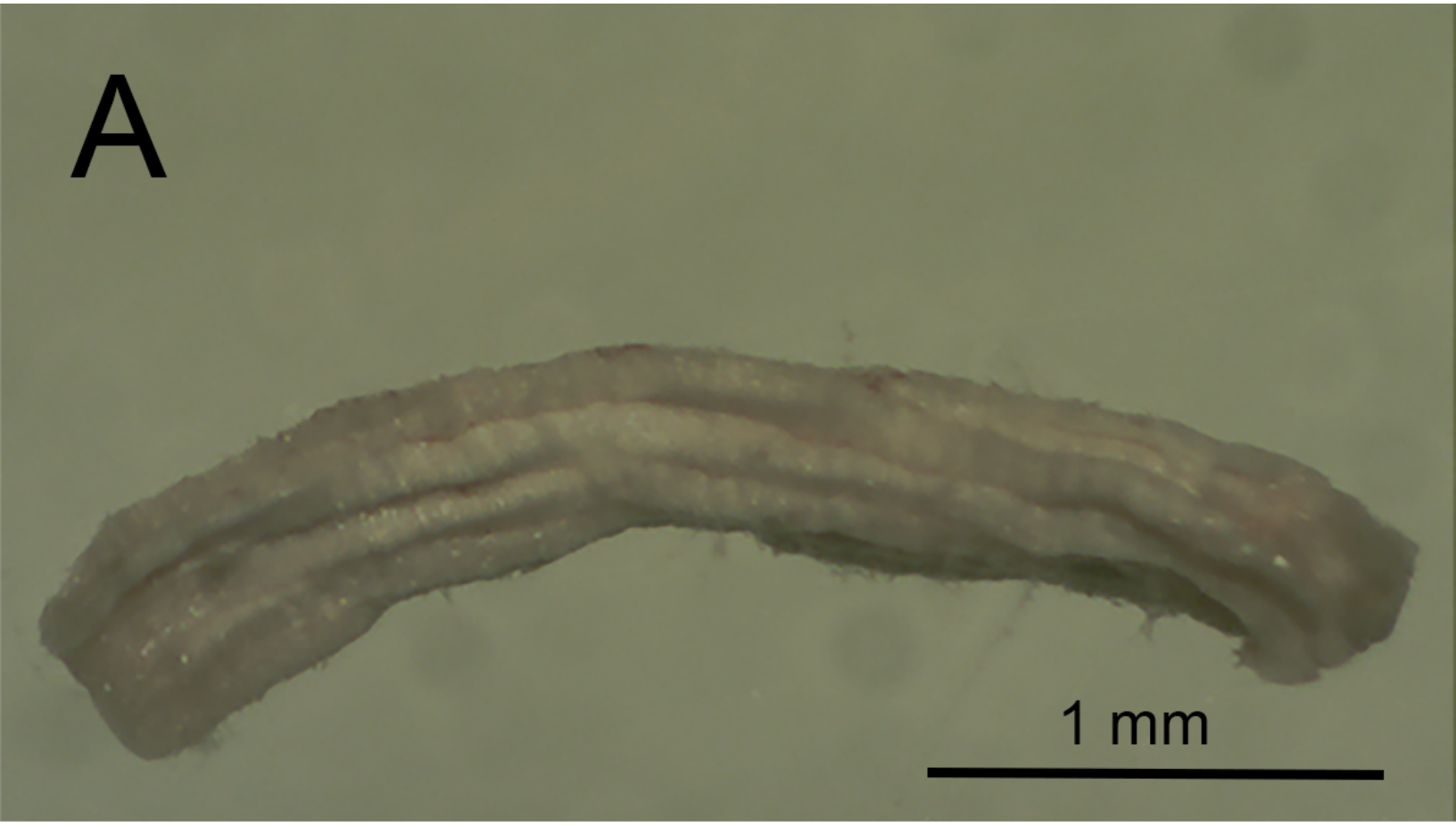
[a] For "Total," data reflect the total amount of protein loaded per lane, as measured by UV exposure of the Criterion gel; For the proteins of interest, data reflect the non-normalised Western blot signal or the normalised protein content.
[b] Arbitrary units (a.u.) are derived from a 4-point calibration curve of mixed-muscle homogenate that was loaded on every gel.
[c] Intraclass correlation coefficient (ICC) ranges were good to excellent for protein content or Western blot signal (i.e., >0.7) and moderate to good for normalised protein content (i.e., >0.5); however, note that the reduction in ICC with normalisation is related to the overall reduction in variance in the dataset (see text).
n = 40 for all datasets; reliability statistics are based on comparisons of the sets of two replicates.
Values in parentheses are 95% confidence intervals.

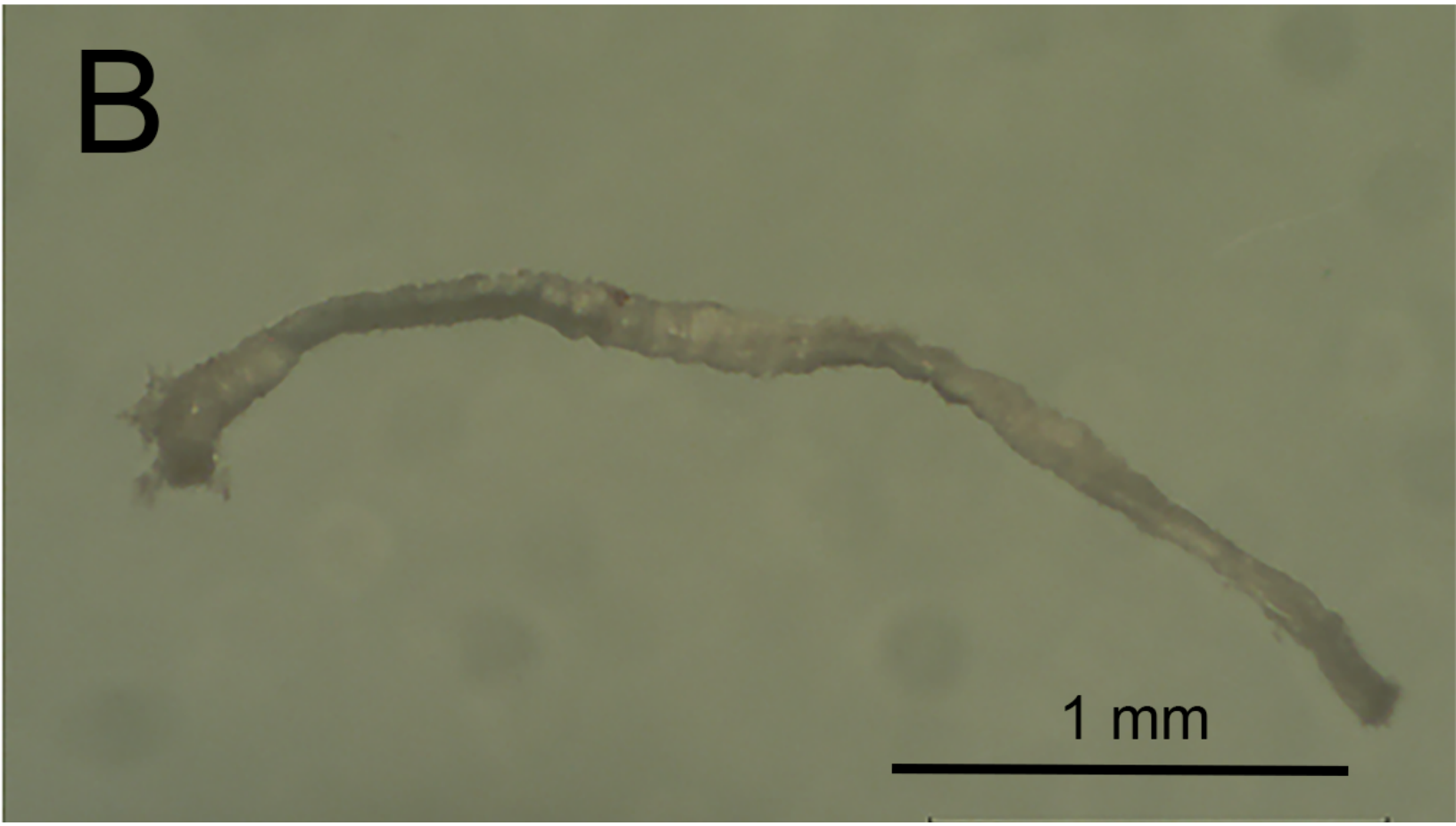
Table 2. The 95% confidence interval widths for normalised protein content estimates derived from a simulated "pooling" of different numbers of type I and type IIa human skeletal muscle fibre segments.

Protein	Fibre type	Mean, a.u. [a]	95% Confidence interval width, a.u. (% of mean) [a] Number of fibres pooled									Break point, a.u. (SE) [a]	Slope, a.u. (SE) [a,b]
			1	5	10	15	20	25	30	35	40		
SERCA2A	I	4.97	4.12 (83)	2.25 (45)	1.56 (31)	1.20 (24)	1.12 (23)	1.03 (21)	0.86 (17)	0.83 (17)	0.82 (17)	6.6 (0.30)	-0.028 (0.002)
SERCA1	IIa	0.94	0.72 (76)	0.45 (48)	0.29 (31)	0.24 (26)	0.21 (22)	0.19 (20)	0.18 (19)	0.17 (18)	0.15 (16)	8.5 (0.33)	-0.005 (0.0004)
AMPKβ2	I	1.08	0.91 (85)	0.42 (39)	0.28 (26)	0.23 (21)	0.20 (19)	0.19 (17)	0.17 (16)	0.16 (14)	0.14 (13)	4.6 (0.24)	-0.006 (0.0005)
	IIa	0.88	0.53 (60)	0.21 (24)	0.15 (17)	0.13 (14)	0.10 (12)	0.10 (11)	0.08 (9)	0.08 (10)	0.08 (9)	3.5 (0.20)	-0.003 (0.0003)
COXIV	I	1.88	1.87 (94)	0.84 (43)	0.60 (30)	0.48 (24)	0.42 (21)	0.36 (18)	0.34 (17)	0.31 (16)	0.29 (15)	4.4 (0.24)	-0.012 (0.001)
	IIa	1.15	1.30 (113)	0.51 (44)	0.35 (30)	0.30 (26)	0.25 (22)	0.22 (19)	0.22 (19)	0.18 (16)	0.18 (15)	3.4 (0.21)	-0.008 (0.0009)

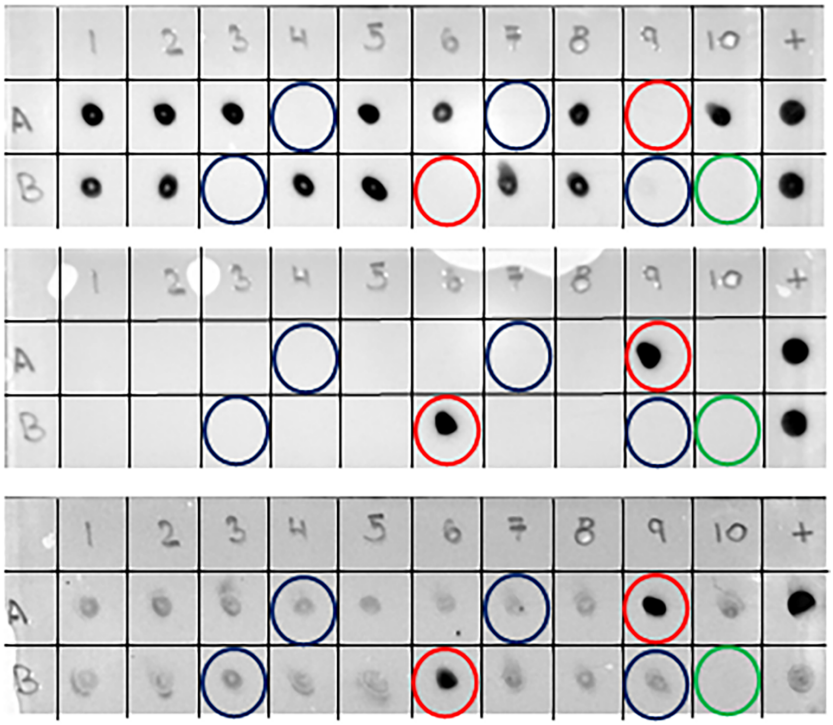
[a] Arbitrary units (a.u.) are derived from a 4-point calibration curve of mixed-muscle homogenate that was loaded on every gel.

[b] The slope of the relationship between the 95% confidence interval width and the number of fibre segments pooled after the breakpoint

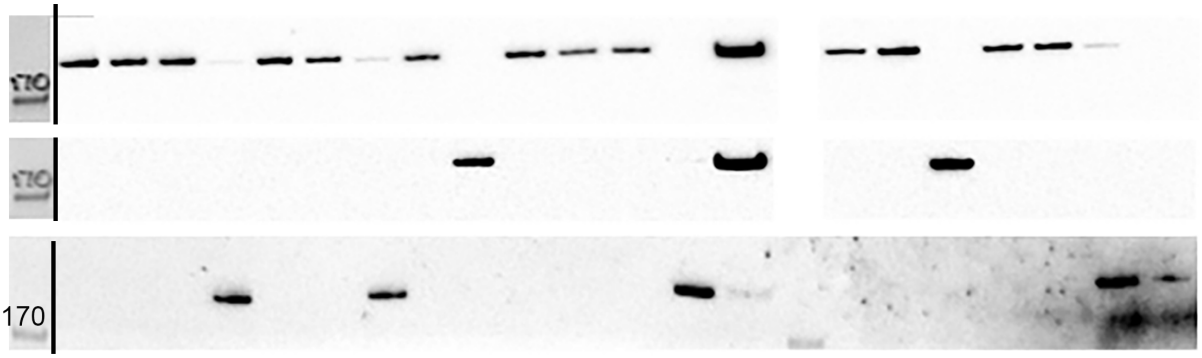

A
1 mm

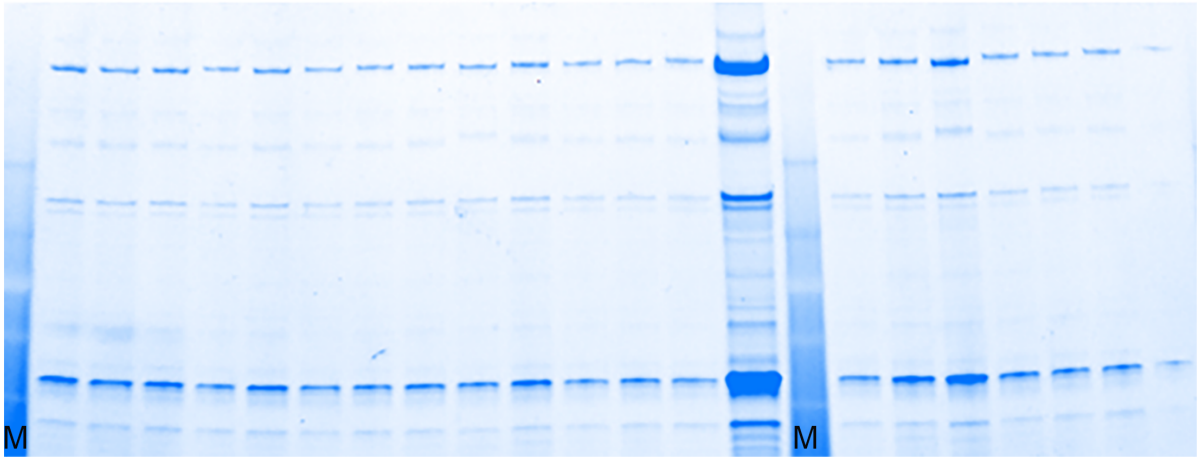

B
1 mm

A



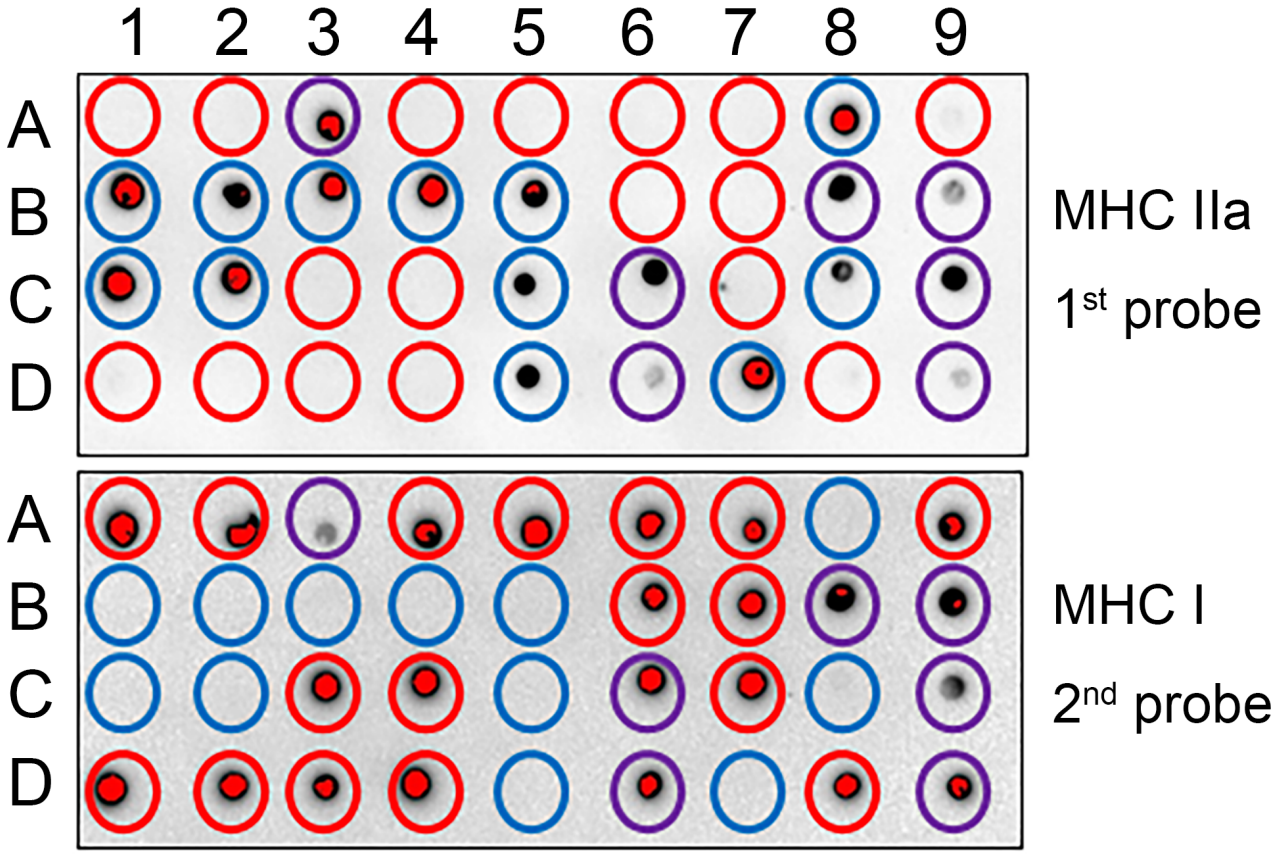
B





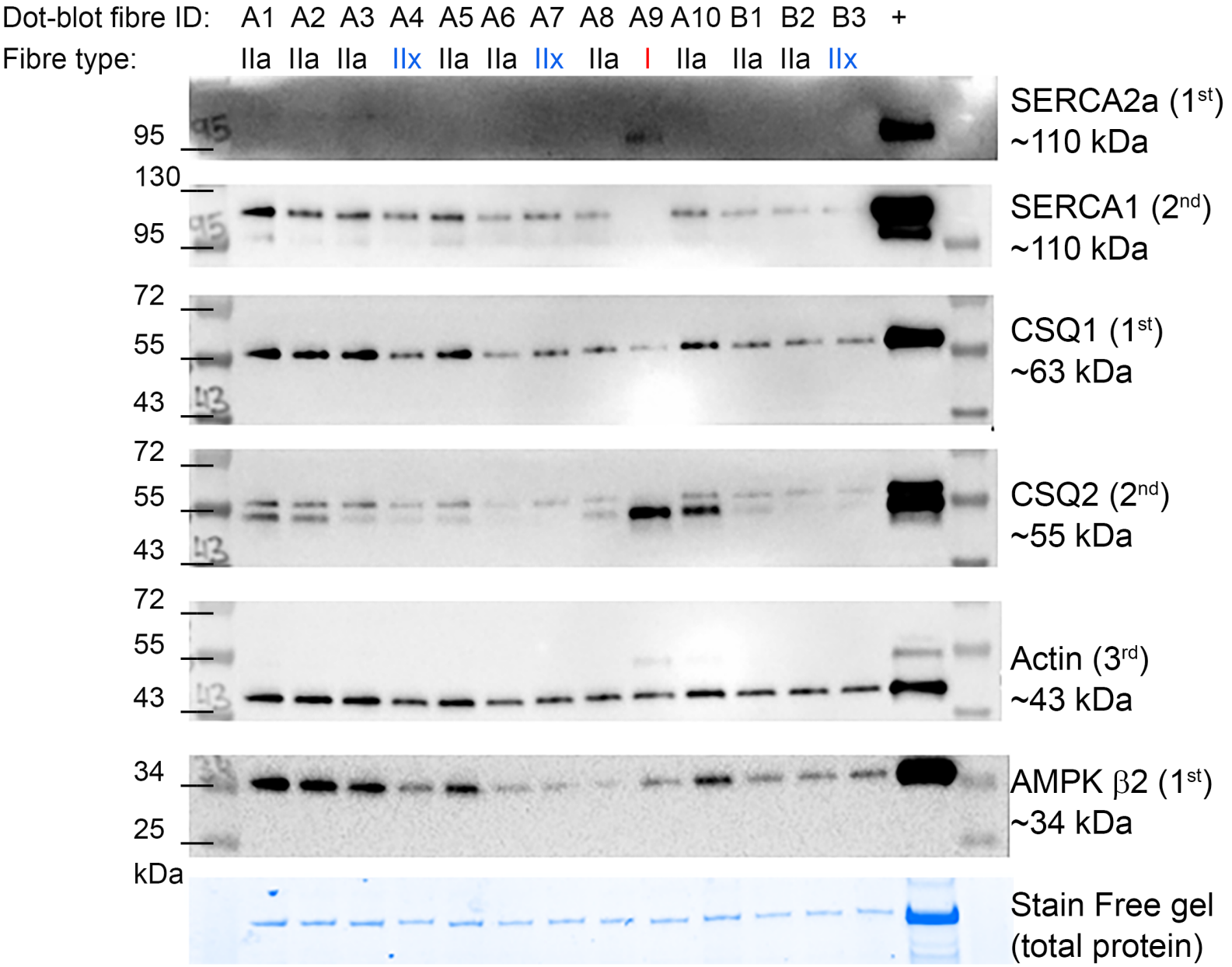
Dot-blot fiber ID:	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	B1	B2	B3	+	B4	B5	B6	B7	B8	B9	B10
Fiber type:	IIa	IIa	IIa	IIx	IIa	IIa	IIx	IIa	I	IIa	IIa	IIa	IIx		IIa	IIa	I	IIa	IIa	IIx	IIx

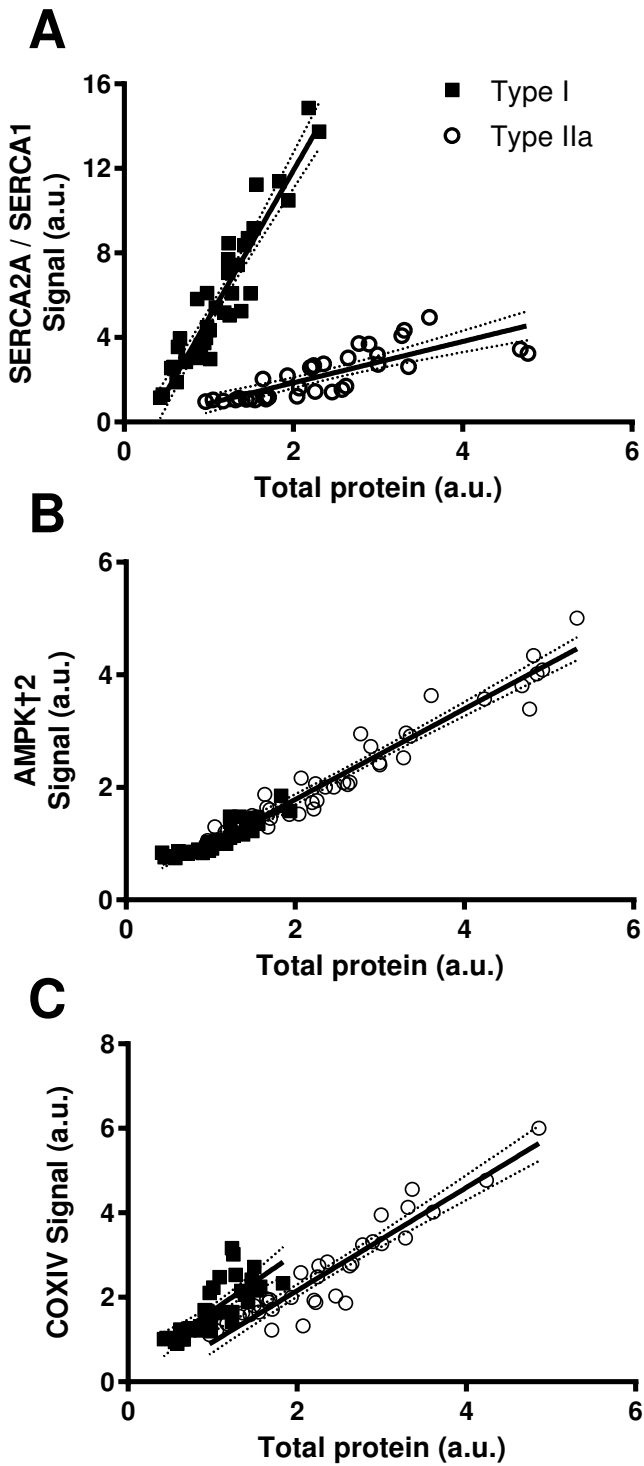
A



B

	1	2	3	4	5	6	7	8	9
A	I	I	I/IIa	I	I	I	I	IIa	I
B	IIa	IIa	IIa	IIa	IIa	I	I	I/IIa	I/IIa
C	IIa	IIa	I	I	IIa	I/IIa	I	IIa	I/IIa
D	I	I	I	I	IIa	I/IIa	IIa	I	I/IIa


Dot-blot fibre ID: A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 B1 B2 B3 +
Fibre type: IIa IIa IIa IIx IIa IIa IIx IIa I IIa IIa IIa IIx
95
130
95
72
55
43
72
55
43
72
55
43
34
25
kDa
SERCA2a (1st) ~110 kDa
SERCA1 (2nd) ~110 kDa
CSQ1 (1st) ~63 kDa
CSQ2 (2nd) ~55 kDa
Actin (3rd) ~43 kDa
AMPK β2 (1st) ~34 kDa
Stain Free gel (total protein)
Dot-blot fibre ID: A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 B1 B2 B3 +
Fibre type: IIa IIa IIa IIx IIa IIa IIx IIa I IIa IIa IIa IIx



Type I (n)
n = 38
Y = 6.99*X - 2.07
R^2 = 0.88

Type IIa (O)
n = 35
Y = 0.98*X - 0.094
R^2 = 0.64

Type I (n)
n = 36
Y = 0.67*X + 0.36
R^2 = 0.82

Type IIa (O)
n = 40
Y = 0.80*X + 0.18
R^2 = 0.94

Type I (n)
n = 35
Y = 1.38*X + 0.31
R^2 = 0.59

Type IIa (O)
n = 35
Y = 1.22*X + 0.28
R^2 = 0.89

Paper V

Christiansen D (2018): Molecular stressors underlying exercise training-induced improvements in K+ regulation during exercise and Na+ ,K+ -ATPase adaptation in human skeletal muscle. Acta Physiol (Oxf). 2019 Mar;225(3):e13196. doi: 10.1111/apha.13196. Impact factor = 5.93.

Received: 25 July 2018 | Revised: 12 September 2018 | Accepted: 28 September 2018

DOI: 10.1111/apha.13196

REVIEW ARTICLE

ACTA PHYSIOLOGICA

Molecular stressors underlying exercise training-induced improvements in K^+ regulation during exercise and Na^+,K^+-ATPase adaptation in human skeletal muscle

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Funding Information
The author was partly supported by an international postgraduate research scholarship from Victoria University, Melbourne, Australia.

Abstract

Despite substantial progress made towards a better understanding of the importance of skeletal muscle K^+ regulation for human physical function and its association with several disease states (eg type-II diabetes and hypertension), the molecular basis underpinning adaptations in K^+ regulation to various stimuli, including exercise training, remains inadequately explored in humans. In this review, the molecular mechanisms essential for enhancing skeletal muscle K^+ regulation and its key determinants, including Na^+,K^+-ATPase function and expression, by exercise training are examined. Special attention is paid to the following molecular stressors and signaling proteins: oxygenation, redox balance, hypoxia, reactive oxygen species, antioxidant function, Na^+,K^+, and Ca^{2+} concentrations, anaerobic ATP turnover, AMPK, lactate, and mRNA expression. On this basis, an update on the effects of different types of exercise training on K^+ regulation in humans is provided, focusing on recent discoveries about the muscle fibre-type-dependent regulation of Na^+,K^+-ATPase-isoform expression. Furthermore, with special emphasis on blood-flow-restricted exercise as an exemplary model to modulate the key molecular mechanisms identified, it is discussed how training interventions may be designed to maximize improvements in K^+ regulation in humans. The novel insights gained from this review may help us to better understand how exercise training and other strategies, such as pharmacological interventions, may be best designed to enhance K^+ regulation and thus the physical function in humans.

KEYWORDS

human skeletal muscle, Ion transport, molecular mechanisms, Na^+-K^+-ATPase, reactive oxygen species, training adaptation

1 | INTRODUCTION

Many important scientific contributions to the topic of potassium ion (K^+) regulation by skeletal muscle have emerged since the discovery in 1938 that the loss of K^+ from excited myocytes is related to altered electrical activity.[1] Many experiments using animals,[2–5] and a substantial number of human studies over the years,[6–9] have consistently shown that the ability to maintain K^+ homeostasis in skeletal muscle is essential for physical function. This is underpinned by observations in both animals and humans that the muscle's capacity for K^+ regulation can be improved by exercise training[7,9] and is reduced in various disease states, including diabetes,[10–15] heart failure,[16] hypertension,[17,18] McArdle disease,[19] and osteoarthritis,[20]

as well as with age,[20–22] inactivity,[23,24] obesity,[13] and caloric restriction.[25] In many of these circumstances, altered K^+ regulation may partly or fully be a consequence of factors arising secondary to the condition. For example, inactivity could be a main factor responsible for the reduced capacity for K^+ regulation with osteoarthritis and age,[23] given the reported lower physical activity level with these conditions.[26,27] In contrast, in systemic diseases, such as hypertension and some types of diabetes, impaired K^+ regulation may, at least in part, arise from the underlying pathophysiological processes. For example, defects in the regulation by insulin of the Na^+,K^+-ATPase, a primary K^+ transport system, could contribute to the abnormal muscle K^+ regulation in insulin-resistant states.[28] Despite this evidence, the molecular mechanisms by which enhancements in skeletal muscle K^+ regulation are mediated have been inadequately addressed in the literature. Identifying these mechanisms is essential because, as the above evidence suggests, they could serve as targets for potential disease-preventive interventions, including pharmacological manipulation. But this information may also be of potential ergogenic value to athletes and their coaches.

Over the past three decades, several excellent reviews have been published highlighting the involvement of K^+ regulation in regulating muscle contractile function, intense exercise performance, as well as its coupling to various disease states.[2,4,14,16,22,29–40] However, most of these reviews go >15 years back and thus lack essential novel inputs, specifically about how different types of exercise training (eg interval-endurance, sprint-interval and resistance training regimens), may affect K^+ regulation and its key determinants, including Na^+,K^+-ATPase function, content, and isoform expression, in humans. In line with findings in animals,[5,41] a number of recent discoveries point to a different regulation of Na^+,K^+-ATPase function and expression by exercise training between different human skeletal muscle fibre types.[23,42–46] However, the potential implications of this fibre type-dependent regulation have been scarcely examined. Furthermore, a number of recent improvements in methodology for muscle protein analysis have been used to investigate changes in Na^+,K^+-ATPase-isoform abundance; experiments that have provided important new insights about the relationship between adaptations in Na^+,K^+-ATPase expression and K^+ regulation in humans that need to be considered.

In this review, I discuss the key molecular mechanisms underpinning improvements in the capacity for K^+ regulation in humans, focusing on skeletal muscle. Then, I provide an update on the effects of different types of exercise training on the skeletal muscle capacity for K^+ regulation and its key determinants, including Na^+,K^+-ATPase function, content, and isoform expression, in humans, with emphasis on fibre-type-dependent adaptations. This is followed by a discussion of how changes in these variables with different types of training may associate with exercise-induced adaptations in mRNA content. On this basis, I examine how to maximize training-induced adaptations in key determinants of K^+ regulation, focusing on blood-flow-restricted exercise as an exemplary model to manipulate the molecular stressors likely required to enhance this capacity in humans.

The synthesis of literature for this review was based on searches in several databases, including PubMed, Web of Science, SportDiscus®, MEDLINE, and Google Scholar. Key search terms used were K^+ regulation, Na^+,K^+-ATPase, FXYD, phospholemman, training adaptation, skeletal muscle, cell culture, myocytes, reactive oxygen species, ion homeostasis, ionic mechanisms, redox state, antioxidant, metabolic stress, mRNA expression, calcium signaling, and AMP-activated protein kinase (AMPK). In addition, manual searches were performed using reference lists from retrieved original studies and review papers.

2 | SITES IN SKELETAL MUSCLE AFFECTED BY PERTURBED K^+ HOMEOSTASIS

At the onset of muscle contraction, rapid and marked perturbations in muscle intracellular and interstitial concentrations of ions (K^+, Na^+, Ca^{2+}, Cl^-, H^+, lac^-) occur, which can both augment and restrict muscle contractile activity.[34,47–51] Strenuous physical exertion raises muscle interstitial K^+ concentration ($[K^+]_{int}$) by up to 2.5- to 3-fold in humans, which has been linked with impaired muscle force development.[6,7,9,48,52,53] This may be caused in part by depolarization of the cell membrane[32,53–55] and altered sarcoplasmic reticulum (SR) Ca^{2+} kinetics (ie decline in rate of SR Ca^{2+} uptake and increased SR Ca^{2+} release),[56] but may also be caused by increased activation of group III/IV afferent nerve fibres by K^+ in the interstitial space, resulting in diminished central motor drive to contracting muscle fibres[57,58] (Figure 1). K^+ efflux from exercising muscles to the bloodstream may also impair myocardial excitation.[59] On the other hand, K^+ acts as a vasodilator and has been proposed to redundantly contribute along with other vasodilatory compounds such as nitric oxide, prostaglandins, and ATP to regulate blood flow to skeletal muscle at rest and during exercise in humans.[60–62] Increased muscle $[K^+]_{int}$ has also been shown to activate the exercise pressor reflex by stimulating afferent nerve fibres, resulting in an elevated heart rate and rate of ventilation.[34,63] These actions can both aid the delivery of substrate for metabolism and the removal of metabolic by-products from exercising or recovering muscle fibres. Altered cytosolic K^+ concentration has also been shown to contribute to the control of mitochondrial ATP synthesis, acting through

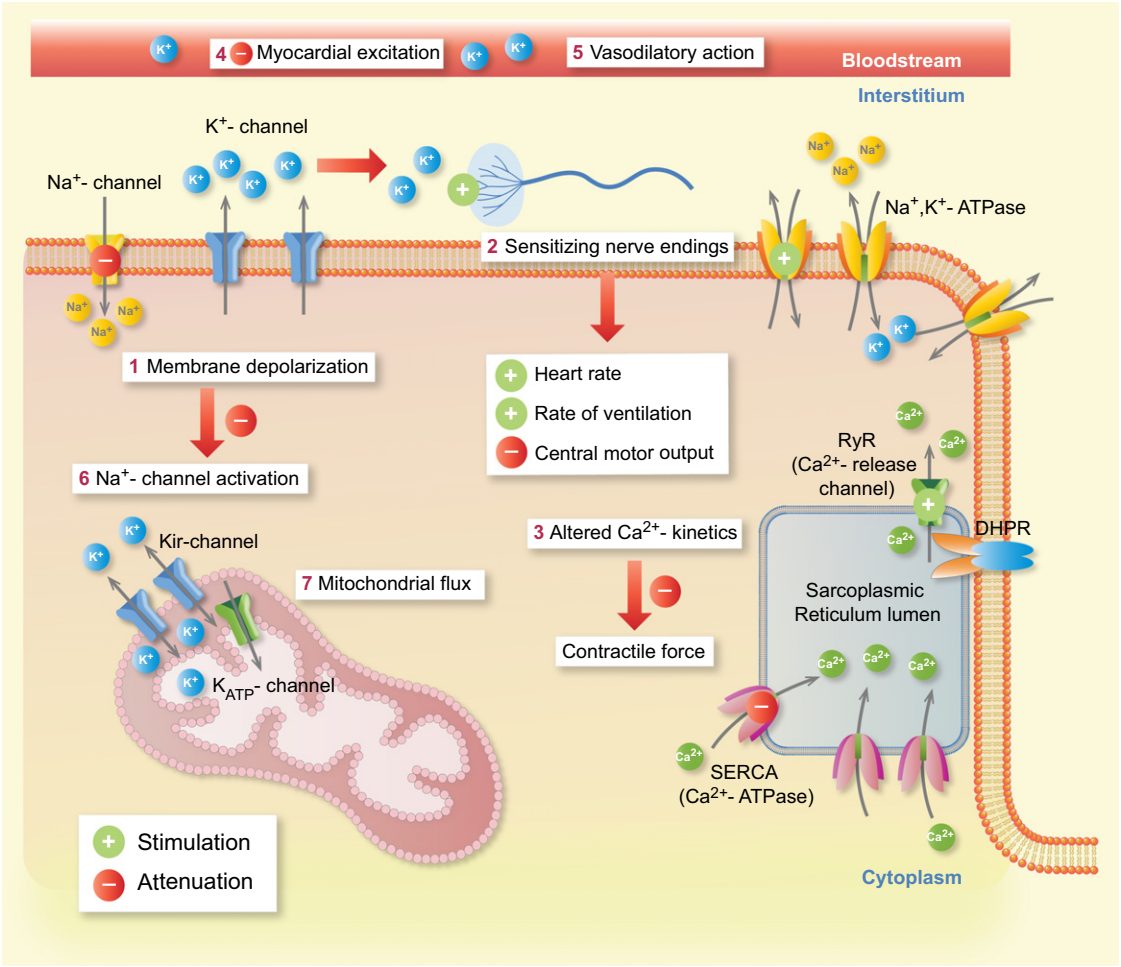

FIGURE 1 Sites in skeletal muscle influenced by elevated interstitial K^+ concentration ($[K^+]_{int}$). Accumulation of K^+ in the interstitial space causes membrane depolarization (1), stimulates group III/IV nerve endings, resulting in decreased central motor output and increased heart and ventilation rate (2), facilitates cytosolic Ca^{2+} accumulation by impairing SR Ca^{2+} re-uptake and enhancing release (3). Increased $[K^+]_{int}$ may also affect myocardial excitation by increasing plasma K^+ concentration (4) and contribute to local vasodilation (5). Furthermore, altered K^+ concentration is known to inactivate Na^+-channels, which is likely to reduce action potential amplitude (6) and impact rate of mitochondrial ATP generation (flux) (7), although its exact role in this matter remains unknown. RyR = ryanodine receptor; DHPR: dihydropyridine receptor

K^+-selective channels localized in the outer and inner mitochondrial membranes, although the exact role of K^+ in the control of mitochondrial oxidative phosphorylation remains debatable.[64] Furthermore, the electrogenic movement of K^+ from the intracellular space to extracellular compartments (interstitium and bloodstream) via voltage-gated K^+ channels likely impacts the transmembrane movement of other ions, including Ca^{2+}, H^+, Na^+, and Cl^-.[65] For example, membrane depolarization invoked by elevated $[K^+]_{int}$ inactivates Na^+-channels, resulting in lowered action potential amplitude.[32] On this basis, proper regulation of K^+ concentrations is essential for the maintenance of both the skeletal muscle contractile function and whole-body metabolic and ion homeostasis and thus physical performance. An overview of the described myocellular sites affected by perturbed K^+ homeostasis and the associated implications for muscle contractile function is provided in Figure 1.

3 | ROLE OF NA^+,K^+-ATPASE ISOFORMS IN REGULATING MUSCLE CONTRACTILE FUNCTION AND ADAPTATION

Intense exercise elicits marked increases (up to ~12 mmol/L) in muscle $[K^+]_{int}$[6,8] and intramyocellular Na^+ concentration,[3] both of which have been linked to muscle fatigue, both directly or indirectly through interactions with other ionic processes.[35] These exercise-induced increases in muscle $[K^+]_{int}$ are rapidly reflected by changes in plasma (arterial and venous) K^+ concentrations, although these changes in absolute terms are of a smaller magnitude.[7,66] In contrast, plasma K^+ concentrations do not appear to be immediately influenced by altered K^+ uptake kinetics in the exercising musculature, as indicated by a dissociation between fluctuations in the net rate of thigh K^+ uptake and

plasma K^+ concentrations during exercise in humans.[7] Exercise-induced ionic (Na^+ and K^+) perturbations in skeletal muscle are primarily counteracted by increasing the activity of the Na^+,K^+-ATPase, which actively transports three Na^+ out and two K^+ into the muscle fibres for each ATP molecule hydrolysed[3,67] (Figure 1). The Na^+,K^+-ATPase is thus critical for maintenance of muscle $Na^+$and K^+ homeostasis, excitability, and contractile function.[32] The Na^+,K^+-ATPase consists of a catalytic α subunit, a structural and regulatory β subunit, and a regulatory γ subunit FXYD, which is coexpressed with the α subunit and is involved in regulating Na^+,K^+-ATPase function, at least in vertebrates.[68–70] In human skeletal muscle, each of the α and β subunits exists as three different isoforms ($\alpha_{1\text{-}3}$ and $\beta_{1\text{-}3}$),[42] whereas FXYD1 (or phospholemman) is the primary isoform of the γ subunit expressed in this tissue.[71] The total capacity for Na^+ and K^+ transport by the Na^+,K^+-ATPase is in part determined by the number of active $\alpha\beta$-heterodimer complexes at the cell surface.[72] But the relative recruitment of different α isoforms may also be influential, because of the distinct ion transport properties of these isoforms.[73,74] Over the past decades, it has been recognized that FXYD also participates in the complex regulation of Na^+,K^+-ATPase function by protecting this system against oxidative damage[75] and by modulating Na^+,K^+-ATPase Na^+affinity via changes in FXYD phosphorylation.[69,76] Nevertheless, the relative contribution of these functions of FXYD in regulating Na^+,K^+-ATPase activity under various conditions (eg, rest and exercise) remains uncertain. The isoforms α_1, α_2, and FXYD1 have been shown in rat skeletal muscles to be translocated from intracellular stores to the cell membrane (only the sarcolemmal portions for FXYD1) in response to exercise and this likely raises Na^+,K^+-ATPase ion transport capacity.[13,77,78] Thus, each of these subunits appears to be functionally relevant and their recruitment important for the net transport of K^+ and Na^+ across the plasma and T-tubular membranes. In addition, the functional Na^+,K^+-ATPase protein complex and its specific isoforms play essential roles in regulating cell volume[79,80] and signaling transduction underlying hypertrophy,[81–83] gene transcription, and protein synthesis,[84] making the Na^+,K^+-ATPase an interesting molecular target in disease therapy, as well as in the context of performance optimization.

4 | MOLECULAR STRESSORS UNDERLYING IMPROVEMENTS IN THE SKELETAL MUSCLE CAPACITY FOR K^+ REGULATION

Exercise demands muscle fibres to contract in a coordinated fashion. This process is mediated by action potential (AP) propagation along sarcolemma and down transverse tubules, where activation of voltage-sensors enables release of Ca^{2+} from the sarcoplasmic reticulum (SR) and resultant initialization of the excitation-contraction coupling. AP propagation is mediated by Na^+ influx, which causes membrane depolarization and K^+ efflux. During the repolarization phase, the positively charged K^+ ions are lost from the fibres through K^+ channels, while influx of chloride ions (Cl-) may also participate in this phase.[32,85] Thus, contracting muscle fibres are exposed to constant perturbations in ion homeostasis. In addition to these perturbations, contracting fibres are under a constant redox disequilibrium due to contraction-induced closure of blood vessels, resulting in episodes with reduced perfusion (ischaemia) separated by periods with reoxygenation. These oscillations in redox homeostasis create a favourable environment for the production of free radicals,[86,87] classified as molecules that contain one or more unpaired electrons.[88] While an imbalance in both ion and redox homeostasis and free radical production have been implicated in the aetiology of muscle fatigue,[85,89] involvement of these processes in training adaptation is an emerging area of research in humans. In this section, the role of these processes in muscle adaptation specific to K^+ regulation, including alterations in expression of Na^+,K^+-ATPase isoforms, will be discussed. Most of our current knowledge on this topic stems from experiments in vitro using cell cultures and animal tissue. These experiments will thus be the center of the following discussion. However, human studies will be included where possible.

4.1 | Oxygenation and redox balance

The severity of fluctuations in local oxygen levels in contracting muscle fibres varies with exercise duration and intensity and partly determine the amount of free radicals that are formed in exercising muscles.[86,90] These redox fluctuations may also exacerbate perturbations in ion homeostasis.[91] Both free radicals[92,93] and disturbance of ion (eg Ca^{2+} and K^+) homeostasis[94,95] have been associated with upregulation of Na^+,K^+-ATPase-isoform expression in vitro. Redox fluctuations could thus be a central determinant of exercise training-induced increases in Na^+,K^+-ATPase abundance and thus K^+ regulation. In rabbit kidney cells, selectively increasing the oxygen level of the cell bathing solution caused an increase in α_1[96] and β_1[96–98] mRNA expression independent of ROS. In another experiment using lung tissue of piglets, increases in both global Na^+,K^+-ATPase mRNA and protein content were evident after breathing hyperoxic gas (inspired oxygen fraction = 0.96).[99] Consistent with the observations in vitro, we have shown that exercise training with reduced muscle blood flow, which substantially raises muscle oxygen perfusion (>3-fold) in the recovery from each exercise bout

(as assessed in vivo by ultrasound Doppler), augmented training-induced increases in muscle Na^+,K^+-ATPase-isoform abundance (β_1 in type I, α_1 in type II, and FXYD1 in both fibre types) concomitant with a reduced net thigh K^+ release during near-maximal exercise in humans.[46] Together, this evidence suggests that increased oxygen perfusion of contracting muscle fibres may be a key stimulus underlying increases in Na^+,K^+-ATPase expression and the capacity for K^+ regulation in human skeletal muscle. In support, an increased oxygen level facilitates transcription of Na^+,K^+-ATPase isoforms in cell culture[96–98] by activating binding of oxygen-sensing transcription factors (specificity protein 1 and 3; Sp1 and Sp3), to promoter regions on Na^+,K^+-ATPase mRNA transcripts.[100,101] However, it should be noted that upregulation of mRNA may be one among several factors that may enhance the potential for an increased net protein turnover of Na^+,K^+-ATPase isoforms in human skeletal muscle, as detailed later in this review. An essential question that arises from this discussion is whether exercise training with a high rate of oxygen consumption, such as endurance-type regimens, would be more beneficial than training with a higher anaerobic ATP turnover for enhancing muscle K^+ regulation. In the second and third part of this review dealing with effects of different types of training and blood-flow-restricted exercise, respectively, this question will be examined in detail.

4.2 | Hypoxia

Because oxygen deficiency (hypoxia) in recruited muscle fibres is an inevitable consequence of the transient closure of blood vessels that occur secondary to muscle contractions, it is also relevant to consider hypoxia as a possible contributory signal to enhancement of K^+ regulation in skeletal muscle. It is well-known that periods with lowered tissue oxygenation can promote ROS production to some degree,[86,102–104] whereas facilitated ROS formation has been linked to increased Na^+,K^+-ATPase expression[92] and K^+ regulation in humans.[105,106] Accordingly, hypoxia enhances the rate of anaerobic ATP turnover during exercise,[91] resulting in an elevated generation of NADPH, an essential substrate for ROS production.[107] Depending on the ROS scavenging capacity of the muscle fibres, hypoxia-induced ROS formation could, under the condition of an inability of the antioxidant systems to scavenge all the accumulating ROS, facilitate S-glutathionylation (ie, oxidative stress) and thus oxidative inhibition of ion channels and transport systems, such as the Na^+,K^+-ATPase,[108] thereby perturbing ion homeostasis. It is also possible that a lowered pH, resulting from the increased rate of anaerobic ATP turnover, could magnify the disturbance of ion homeostasis by affecting the function of ion transport systems and channels.[109] Such ionic perturbations may favor Na^+,K^+-ATPase-isoform synthesis, in part by activation of Ca^{2+}-signaling pathways (eg, via the Ca^{2+}/calmodulin-dependent protein kinase, CaMK and calcineurin) and mRNA transcription.[91,94,95] Given these observations, It may seem surprising that different types of hypoxic training concepts, including exercising in normobaric, systemic hypoxia[110,111] and living at high and training at low altitude (LHTL),[112–114] either decreased or had no effect on, respectively, muscle Na^+,K^+-ATPase-isoform abundance or plasma K^+ concentration during exercise in humans. We recently assessed in humans the role of muscle hypoxic level per se on the molecular signaling events thought to be involved in improving muscle K^+ regulation by exercise.[91] In this study, we compared changes in mRNA and activation of signaling proteins (ie AMPK and CaMKII) to exercise sessions performed with reduced muscle blood flow (ie, blood flow restriction; BFR) and in systemic hypoxia (~3250 m altitude). Key observations were that BFR augmented exercise-induced increases in FXYD1 mRNA content, type-I fibre AMPK downstream signaling (increased ACC phosphorylation), and in markers of oxidative stress,[91] consistent with an elevated FXYD1 protein abundance and a reduced net thigh K^+ release (ie improved K^+ regulation) during exercise following 6 weeks of blood-flow-restricted training.[46] In contrast, the session in systemic hypoxia did not result in selective changes in levels of Na^+,K^+-ATPase-isoform mRNA transcripts, AMPK or CaMKII downstream signaling, or oxidative stress, despite a similar level of muscle hypoxia (as assessed in vivo by near-infrared spectroscopy; NIRS) compared to the session with BFR.[91] Importantly, the measurement of muscle oxygen level in the latter study likely accounted for a possible compensatory rise in muscle oxygen perfusion that is likely to occur by exercising in systemic hypoxia (ie arterial hypoxaemia),[115] but is unlikely to be present to the same degree during exercise with BFR where blood flow is mechanically restricted by a cuff. However, it should be noted that the use of NIRS does not take into account local spatial and temporal oscillations in oxygen perfusion that may take place in exercising muscles.[116]

Together, these findings suggest that hypoxia per se is not an essential signal for increasing Na^+,K^+-ATPase expression and K^+ regulation by exercise in human skeletal muscle. In agreement, long-term exposure to hypoxia, such as during live-high and continuous systemic-hypoxia training regimens, is not beneficial to these adaptations. On the other hand, repeated, transient muscle exposures to hypoxia, as induced by interval training with BFR, appear to promote these adaptations in humans. Accordingly, molecular alterations that may occur secondary to the repeated episodes of muscle hypoxia during exercise, such as repeated, transient increases in anaerobic ATP turnover and perturbed ion (K^+ and Ca^{2+}) homeostasis, could be important

contributing factors for enhancing K^+ regulation and Na^+,K^+-ATPase adaptation in human skeletal muscle, as discussed later in this review.

4.3 | Reactive oxygen species

Reactive oxygen species (ROS) is a broad term used to classify oxygen-centered molecules that contain one or more unpaired electrons, but also includes reactive derivates of oxygen such as hydrogen peroxide (H_2O_2). While many ROS thus can be defined as free radicals, this term also covers reactive nitrogen species (RNS), which refers to free radicals for which nitrogen is the reactive center.[88] For a thorough inspection of the different types and sources of ROS in skeletal muscle, the reader is referred to the reviews by Powers, Ji, Kavazis, Jackson[88] and Jackson, Pye, Palomero.[117] It is now well-established that chronic increases in ROS levels are involved in the aetiology of many pathological conditions, including type-II diabetes[118] and peripheral artery disease.[119,120] Conversely, transient increases in ROS levels that are rapidly reversible are central to the regulation of normal contractile function,[121] as well as signaling transduction underlying training adaptation.[122,123] The latter roles of ROS in relation to K^+ regulation will be the focus of the following discussion.

Exercise elicits marked increases in ROS concentrations in skeletal muscle, which can modulate muscle force development in both a time- and dose-dependent manner.[124] High doses of ROS have been shown to impair myocytic force development by perturbing ion (eg Ca^{2+} and K^+) homeostasis.[121,125] Accordingly, antioxidant treatment in humans attenuated exercise-induced increases in arterialised-venous K^+ concentration,[105,106] and thigh K^+ release,[46] indicating ROS may affect plasma and muscle K^+ homeostasis in exercising humans. Disturbance of K^+ homeostasis due to ROS accumulation is likely mediated via oxidative modifications to, and in this case dysfunction, of K^+ channels and transport systems, including the Na^+,K^+-ATPase.[90,121,126–128] One type of modification is the formation of disulphide bonds between glutathione and reactive cysteine thiols on amino acid structures (S-glutathionylation), ie oxidative damage.[129,130] Na^+,K^+-ATPase dysfunction induced by severe ROS formation has been demonstrated in cell culture preparations,[108,131–133] and this has been confirmed by observations of an inverse relationship between the degree of glutathionylation of Na^+,K^+-ATPase subunits and Na^+,K^+-ATPase activity in rat skeletal muscles[129] and findings of increased β-subunit glutathionylation coinciding with fatigue during intense exercise in humans.[130] However, the observation that Na^+,K^+-ATPase is under redox control is not new. The first evidence for such regulation was published in the sixties, where hydrogen peroxide (H_2O_2) treatment markedly depressed Na^+,K^+-ATPase activity in electrical eels.[134] Later, this was confirmed in several other tissues, including the brain,[135] kidney,[136,137] and myocardium [138,139] by actions of the hypochlorite anion, hydroxyl radicals, superoxide, and singlet oxygen. Around the same time, it was shown that Na^+,K^+-ATPase activity is inhibited by tert-butyl hydroperoxide at high, but not at low concentrations,[140] and a dose-dependent (inverse U-shaped) effect of ROS on Na^+,K^+-ATPase function was documented in rat cerebellar granule cells a decade later.[128] This relationship is strikingly similar to that reported for muscle contractile function by Lamb, Westerblad.[121] Thus, tight control of ROS levels seems necessary to preserve K^+ homeostasis and contractile performance of skeletal muscle. But equally important, this evidence underscores that the degree and pattern of ROS accumulation in contracting fibres could play a central role in regulating adaptations specific to K^+ regulation in part by affecting ion homeostasis.

In addition to their acute impact on ion (eg K^+, Na^+ and Ca^{2+}) handling systems, ROS may be involved in the long-term (chronic) regulation of muscle Na^+,K^+-ATPase expression by exercise training via actions as signalling transducers for mRNA transcription and protein synthesis.[92,122,141] In humans, involvement of ROS in regulating the turnover of mRNAs of importance to ion transport function has, to my knowledge, only been investigated (indirectly) in a single study. In this study, Murphy, Medved, Brown, Cameron-Smith, McKenna [92] found that intravenous infusion with the multiple ROS scavenger, N-acetylcysteine, blunted the rise in Na^+-K^+-ATPase α_2-isoform mRNA during the recovery from 45 minutes of cycling at 71% VO_{2max}. Furthermore, they observed that pre-incubation of rat EDL muscle with N-acetylcysteine abolished the increase in Na^+-K^+-ATPase α_1, α_2 and α_3 mRNA induced by electrical stimulations in vitro. Although the impact of N-acetylcysteine on muscle antioxidant status was not determined in that study, it demonstrates that ROS accumulation may be a critical determinant of exercise-induced increases in mRNA content of catalytic Na^+-K^+-ATPase isoforms in mammalian muscles. In agreement, long-term treatment of kidney cells with H_2O_2 resulted in upregulation of Na^+-K^+-ATPase expression and activity, whereas adding a bolus of the antioxidant apocynin abolished these effects in vitro.[93] In extension of these results, blood flow restriction has been shown to augment increases in oxidative damage to a single exercise session,[91] as well as facilitate training-induced improvements in Na^+-K^+-ATPase-isoform protein abundance and K^+ regulation in human skeletal muscle.[46] Adding to this point, rapid and marked perturbations in redox homeostasis effectively increase ROS levels,[142–147] whereas a single bout of post-exercise cold-water immersion, which is likely to temporarily perturb muscle redox state, caused

a selective increase in Na^+-K^+-ATPase α_2 isoform mRNA expression in skeletal muscle of recreationally-active men.[45]

Thus, increased muscle ROS production and resultant oxidative stress during training sessions may be an important stimulus for training-induced enhancements in skeletal muscle Na^+,K^+-ATPase-isoform content and its capacity for K^+ regulation in humans. This conclusion is underlined by observations that oxidative damage to ion channels and transport systems, such as the Na^+,K^+-ATPase, exacerbates disturbances in ion homeostasis,[75,108,126] which has been coupled to elevated expression of Na^+,K^+-ATPase isoforms.[94,95] Accordingly, in humans, greater exercise-induced changes in venous plasma K^+ concentration were associated with a more pronounced mRNA and signaling response underlying adaptations specific to K^+ regulation.[91]

4.4 | Antioxidant function

To counteract oxidative damage during periods of elevated ROS exposure, muscle fibres contain a network of strategically-located ROS scavenging systems, including enzymatic and non-enzymatic antioxidants, defined as substances that either delay or hinder oxidation of a substrate.[88] The primary antioxidant enzymes are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). These enzymes catalyse the degradation of highly-reactive ROS into less-reactive molecules. Non-enzymatic antioxidants also provide a significant reservoir for ROS scavenging and this term covers substances such as glutathione, uric acid, bilirubin, biliverdin, and carnosine, among others.[148–151] The content and function of these scavengers are typically altered by exercise training. In humans, for example, exercise training regardless of the type being performed (eg endurance, resistance, or a combination) has been reported to increase the activity and/or expression of SOD, GPx, glutathione:glutathione disulphide ratio, total antioxidant capacity, and/or decrease oxidative damage in blood, erythrocytes, or skeletal muscles.[152–155] Antioxidant treatment acutely reversed ROS-induced oxidative inhibition of ion channels and transport systems in vitro,[126] whereas an improved K^+ regulation after a period of blood-flow-restricted training was temporally linked with altered muscle antioxidant capacity in humans.[46] Furthermore, expression of the cytosolic (copper/zinc) isoform of the antioxidant enzyme superoxide dismutase (SOD1) increased, concomitant with increases in Na^+,K^+-ATPase α_1, β_1, and FXYD1 abundance, in the vastus lateralis muscle of humans after 15 weeks of swimming.[156] These findings support that antioxidant function could serve as a fine-tuning system in the regulation of K^+ homeostasis in skeletal muscle by controlling levels of ROS and this possible interaction may be altered by exercise training. More research in humans is required to shed more light on this topic. Given ROS have been linked with the aetiology of several diseases, including diabetes [118] and peripheral artery disease,[120] an essential question that remains to be answered in this regard is whether an impaired capacity for K^+ regulation associated with such diseases [12,157] may be circumvented by modulating antioxidant function.

In summary of the last two sections, the level of ROS appears to be a critical factor in both the acute and long-term regulation of K^+ transport function and Na^+,K^+-ATPase expression in skeletal muscle. As only few studies have been undertaken using human subjects and these did not directly assess ROS levels, present conclusions concerning a role of ROS in regulating adaptations specific to K^+ regulation in humans are currently restricted to indirect measurements. Furthermore, enhancements in antioxidant function may be a contributory explanation for exercise training-induced enhancement (or fine-tuning) of K^+ regulation in humans. Future human studies are warranted to elucidate the extend to which different sources of ROS and antioxidants may play a role in the adaptive response related to altered K^+ regulation by exercise training (and other interventions) in human skeletal muscle.

4.5 | Intracellular Na^+

The first evidence to support a role of intracellular Na^+ ($[Na^+]_i$) in mediating increases in the abundance of Na^+-K^+-ATPase subunits stems from studies using cultured rat cardiac and vascular smooth muscle cells published in the early nineties. In these reports, a rise in $[Na^+]_i$ facilitated by culture incubation with aldosterone,[158] thyroid hormone,[159] ouabain (an inhibitor of the Na^+-K^+-ATPase) [160] and veratridine (a Na^+-channel activator) [161] induced a 2- to 7-fold increase in the mRNA expression of the Na^+-K^+-ATPase isoforms. A potent role of $[Na^+]_i$ was later confirmed by findings of increased α_1 and β_1 mRNA in rat kidney epithelial cells and astrocytes incubated with ouabain.[162,163] At this time, it therefore seemed plausible that $[Na^+]_i$ could be a key initiator of Na^+-K^+-ATPase gene transcription. But Murphy, Macdonald, McKenna, Clausen [94] later observed that incubation with ouabain (2 h), veratridine (30 minutes), or monensin, a Na^+ ionophore/carrier (30 minutes), abolished the increase in Na^+,K^+-ATPase α_1, α_3, β_1 and β_3 mRNA in rat EDL muscle after intermittent electrical stimulations in vitro. They also observed a decline in α_1 and β_2 mRNA content with ouabain and veratridine, whilst all incubations caused a downregulation of β_3. Thus, a chronic high $[Na^+]_i$ might not be beneficial, and could even be detrimental, to the adaptability of the Na^+-K^+-ATPase genes in mammalian skeletal muscle. In contrast, the intermittent electrical stimulations increased

the catalytic isoforms (α_1, α_2 and α_3).[94] This suggests that an oscillatory nature of $[Na^+]_i$ could be a potent stimulus for elevating Na^+-K^+, ATPase mRNA expression. This is in agreement with a 1.4- to 3.4-fold increase in the α_1-, α_2- and α_3-isoform expression in human muscle in recovery from repeated intense contractions,[164,165] which is known to induce drastic fluctuations in $[Na^+]_i$. In addition, Na^+-induced Na^+-K^+, ATPase signal transduction is linked to elevated ROS production,[84] which could facilitate Na^+,K^+-ATPase-isoform mRNA transcription,[92] highlighting a possible mechanistic link between increased $[Na^+]_i$ and Na^+,K^+-ATPase expression. More research into the possible role of $[Na^+]_i$ in regulating adaptations in Na^+-K^+-ATPase expression and K^+-transport function in human muscle is clearly required.

4.6 | Extracellular K^+ and membrane depolarization

In isolated rat EDL muscle, increasing $[K^+]$ (13 mmol/L) of the muscle bathing solution invoked an increase (160%) in Na^+,K^+-ATPase α_1 mRNA content.[94] This suggests that membrane depolarization, as a result of the increased extracellular $[K^+]$, may be a potent stimulus for increasing muscle α_1 mRNA content. In agreement, inhibition of Na^+,K^+-ATPase activity by adding 0.5 to 1.0 mmol/L ouabain, which increases extracellular $[K^+]$,[3] raised α_1 and β_1 mRNA content in astrocytes in vitro. Similar effects of depressed Na^+,K^+-ATPase activity have been observed in humans, where increases in α_1 (1.5 fold) and α_2 mRNA (2.5 fold) after fatiguing knee-extensor exercise were inversely correlated with the change in 3-O-MFPase activity from rest to exhaustion ($r = -0.60$ in both cases; $P < 0.05$).[166] Consistent with this, more pronounced changes in venous plasma K^+ concentration during training sessions caused by blood flow restriction were associated with greater training-induced improvements in skeletal muscle K^+ regulation and exercise performance in humans.[46,91] Taken together, these observations indicate that extracellular K^+ accumulation and resultant membrane depolarization positively regulates Na^+,K^+-ATPase-isoform expression and K^+ regulation in the musculature, although more studies are necessary to confirm the scarce number of observations in human skeletal muscle.

4.7 | Cytosolic Ca^{2+}

In mouse muscle, it has been shown that Ca^{2+} is released from SR in an exercise-intensity dependent manner, which appears to be tightly coupled to activation of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in intact muscle fibres.[167] Exercise-induced induction of some Na^+, K^+-ATPase mRNA transcripts is positively associated with exercise intensity (eg isoform α_1, Figure 2). Thus, it is possible that fluctuations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and altered activation of CaMKII could play a role in exercise-induced changes in Na^+-K^+-ATPase-isoform mRNA content in skeletal muscle fibres. This was investigated by Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard.[168] Based on rat muscle incubations in vitro, they found the Na^+,K^+-ATPase α_1-isoform to be regulated by Ca^{2+} signalling pathways (CaMK and calcineurin). In another in vitro rat experiment, a rise in $[Ca^{2+}]_{cyt}$ induced by incubation with the Ca^{2+} ionophore A-23187 elevated the Na^+-K^+-ATPase α_3 mRNA (1.2 fold), but reduced β_1 (0.8 fold). Thus, despite a scarcity of published research, it appears likely that increases in $[Ca^{2+}]_{cyt}$ and resultant signalling transduction through either CaMKII, calcineurin, or both, may be involved in exercise-induced regulation of Na^+-K^+-ATPase-isoform mRNA levels in skeletal muscle. However, exercise-induced modulation of the degree of phosphorylation at Thr^{287}, and thus autonomous activity,[169–171] of CaMKII was dissociated from increases in Na^+-K^+-ATPase α_2 and FXYD1 mRNA in human skeletal muscle,[91] indicating signaling via CaMKII is not required for exercise-induced increases in the levels of some Na^+,K^+-ATPase mRNA transcripts in human skeletal muscle. Nevertheless, CaMKII activity has been shown to be upregulated at the onset of exercise, from where it may gradually decrease towards resting level during sustained moderate-intensity

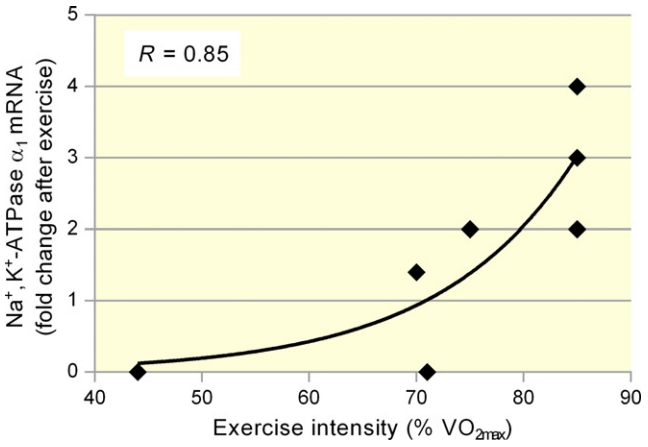

FIGURE 2 Effect of exercise intensity on change in Na^+,K^+-ATPase α_1 mRNA in response to a single session of exercise in human skeletal muscle. The figure is based on data from Aughey, Murphy, Clark, Garnham, Snow, Cameron-Smith, Hawley, McKenna [165] and Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard [95] that used similar cohorts (trained humans) and exercise modality (cycling). A single, two-parameter exponential fit [f = a(exp (bx))] provided the strongest relationship between the two factors ($r = 0.85$)

exercise.[170] As such, the timing of the muscle biopsy after the end of the exercise session in the latter study may not have been optimal for detecting changes in CaMKII phosphorylation (and activity). Thus, further experiments in humans are warranted to clarify the involvement of altered $[Ca^{2+}]_{cyt}$ and activation of Ca^{2+} signaling proteins in mediating Na^+-K^+-ATPase adaptation.

4.8 | Anaerobic ATP turnover and the 5'AMP-activated protein kinase

Among the few published human studies, there is consensus that exercising with a high, compared to a lower, relative intensity yields a more powerful stimulus for induction of ion transport genes.[164,168,172] This is supported by the positive relationship ($r = 0.85$) between exercise intensity and fold-increases in muscle Na^+-K^+-ATPase α_1-isoform mRNA in trained humans (Figure 2). Sustained exercise at a high intensity requires a high anaerobic energy turnover, which results in accumulation of H^+ (ie decline in pH) in exercising muscles. In turn, this has been shown to impair the function of K^+ and Ca^{2+} transport systems, including the Na^+-K^+-ATPase and SR Ca^{2+}-ATPase (SERCA),[173,174] thereby exacerbating perturbations in ion homeostasis in the exercising musculature. However, this pH dependency remains to be shown in human skeletal muscle within the physiological range of fluctuations in muscle (or intracellular) pH. Furthermore, a marked increase in anaerobic glycolytic flux raises the availability of NAD(P)H for ROS production and exacerbate fluctuations in redox homeostasis.[175] On this basis, the degree of anaerobic energy turnover may be an important determinant of the effects of a given exercise intervention on the expression of, at least some, Na^+-K^+-ATPase isoforms. Accordingly, decreasing the relative exercise intensity by performing the same exercise session after compared to before a period of intense training attenuated the exercise-induced increase (threefold) in α_1 mRNA observed before training.[164] However, several other factors might be involved in this regulation. For example, exercise has been shown to activate AMPK in an intensity-dependent manner,[176] whereas increased activity of AMPK has been linked with transcription of both the Na^+-K^+-ATPase β_1 isoform and FXYD1 in vitro.[177] In support of these results, the degree of AMPK downstream signaling (ie phosphorylation of the Acetyl-CoA carboxylase, ACC) was positively associated with upregulation of FXYD1 mRNA in human skeletal muscle.[91] This provides an indication of involvement of AMPK in mediating adaptations in expression of FXYD1 in human muscle. However, although phosphorylation of ACC strongly reflects AMPK activity,[178] it may be elevated by factors other than AMPK. Thus, at present, it cannot be unequivocally stated that AMPK is involved in regulating FXYD1 expression in human muscle. Further studies are warranted to determine the relationship between exercise-stimulated changes in muscle AMPK activity and FXYD1 abundance in humans.

4.9 | Lactate

The concentration of lactate, a surrogate marker of anaerobic ATP consumption, has been shown to regulate the expression of several mRNA transcripts involved in various cellular functions, including PGC-1α, a regulator of mitochondrial content.[179] We recently examined whether lactate accumulation could be a regulator of the acute molecular response underpinning adaptations in K^+ regulation to exercise in humans.[91] This was done by modulating muscle lactate concentration during exercise using blood flow restriction and systemic hypoxia, both types of exercise shown to invoke drastic increases in muscle lactate concentration.[180,181] Despite similar muscle lactate concentration, the exercise sessions differed with respect to alterations in expression of Na^+-K^+-ATPase mRNA transcripts,[91] indicating elevated muscle lactate does not play a direct role in regulating exercise-induced adaptations in mRNA expression of Na^+-K^+-ATPase isoforms in human skeletal muscle. This finding is limited to the mRNA level, thus further research is required to clarify if lactate accumulation during training sessions is an important determinant of (long-term) adaptations in the function and content of the Na^+-K^+-ATPase, and ultimately of improvements in muscle K^+ regulation, in humans, although this would seem unlikely based on the existing evidence.

4.10 | Summary

In Table 1, an overview of the molecular stressors potentially involved in mediating adaptations specific to K^+ regulation in skeletal muscle is provided, along with an indication of the empirical support from animal/cell culture (in vitro) and human experiments provided to each of these stressors about their involvement in mediating these adaptations. In summary, improvements in K^+ regulation and increases in expression of Na^+-K^+-ATPase isoforms in human skeletal muscle are likely initiated by transient perturbations in redox, ionic, and metabolic state, whilst increased oxygenation, ROS levels, extracellular $[K^+]$, and anaerobic ATP turnover may be of particular importance to this regulation. In contrast, the level of muscle hypoxia and lactate concentration per se do not appear to be essential to these adaptations in humans. At present, the involvement of alterations in $[Na^+]_i$ and $[Ca^{2+}]_{cyt}$ is unclear, although evidence in vitro points to a role of transient shifts in the concentrations of these ions in increasing Na^+-K^+-ATPase expression. Little is currently understood about how the identified key molecular stressors may be conveying the

TABLE 1 Summary of the likely involvement of key molecular stressors in mediating adaptations specific to K^+ regulation in human skeletal muscle

Molecular signal	Interaction with other key signal mechanisms	Effect on adaptation in K^+ regulation or Na^+,K^+-ATPase expression	Evidence in animals or cell culture	Supported by findings in humans
Perturbations in redox state	↑ ROS, altered ion homeostasis	+	Yes	Yes
↑ oxygenation	↑ Sp1/Sp3 activation; ↑ mRNA transcription	+	Yes	(Yes)
Hypoxia	Redox homeostasis	–	Yes	Yes
↑ ROS levels	↑AMPK activation, perturbed K^+, Ca^{2+}, Na^+ homeostasis	+	Yes	Yes
↑ Antioxidant function	↓ ROS	–	Yes	Yes
Intracellular Na^+	Ion homeostasis, ↑ ROS	?	Conflicting	n/a
Interstitial K^+ accumulation (membrane depolarization)	Ion homeostasis, ↑ ROS	+	Yes	No
Cytosolic Ca^{2+}	Ion and redox homeostasis, CaMKII activation	+	Yes	No
Anaerobic ATP turnover	AMPK, perturbed ion and redox homeostasis, ↑ ROS	+	No	(Yes)
Lactate	Metabolic homeostasis	–	No	Yes

↑ and ↓ denote potentiation and attenuation respectively. + and – denote a positive and negative effect respectively. ? denotes unknown and brackets denote weak support.

signal(s) for adaptation in the capacity for K^+ regulation in human skeletal muscle, although some evidence points to a contributory role of AMPK and oxygen-sensing transcription factors.

5 | AN UPDATE ON THE EFFECTS OF EXERCISE TRAINING ON K^+ REGULATION, AND NA^+,K^+-ATPASE FUNCTION, CONTENT, AND ISOFORM ABUNDANCE, IN HUMAN SKELETAL MUSCLE

In the previous section, the key molecular stressors underpinning improvements in the capacity for K^+ regulation in humans were reviewed. It is obvious to speculate that strategies, such as exercise training, that have the potential to substantially promote these stressors could be useful to enhance K^+ regulation and physical function in humans. In the next sections, an update on the effects of different types of exercise training on key determinants of K^+ regulation in humans, including plasma K^+ concentration, thigh K^+ release, and skeletal muscle Na^+,K^+-ATPase activity, content, and isoform abundance, is provided, and findings on the fibre-type-dependent regulation of Na^+,K^+-ATPase-isoform abundance will be discussed. Then, it is examined how changes in these variables with different types of training may relate to exercise-induced adaptations in mRNA expression.

From the cross-sectional data in Table 2 and the visual summary provided in Figure 3, it is clear that exercise training, regardless of whether it is performed at/below (primarily aerobic; AEH) or above (primarily anaerobic; ANH) the intensity eliciting VO_{2max}, is an effective stimulus to increase both Na^+,K^+-ATPase content (11-15%; as determined by [^{3}H]-ouabain binding) and isoform abundance (5-44%; as quantified using western blotting) in humans. Training with a high aerobic energy component (AEH) has also been shown to increase Na^+,K^+-ATPase maximal in vitro activity (4%; as assessed by the 3-O-MFPase method), whereas the effects of ANH training modalities on this variable remain to be examined. The functional relevance of increases in Na^+,K^+-ATPase content, isoform abundance, and/or activity is evidenced by findings of concomitant reductions (5-8%) in K^+ concentration in the bloodstream or in the muscle interstitial space during exercise, and simultaneous improvements (14-16%) in physical performance in many studies (Table 2). These adaptations may occur rapidly, with decreases in venous blood [K^+] (5%) and increases in Na^+,K^+-ATPase activity (41%), content (9-14%), and isoform abundance (27-113%, α_1, α_2 and β_1), reported after only six to ten days of training.[182–184] However, changes in functional variables, such as thigh K^+ release, venous [K^+], and Na^+,K^+-ATPase activity, with both one type or different types of training appear to be dissociated from those of Na^+,K^+-ATPase content or isoform abundance measured in whole-muscle

TABLE 2 Effects of different types of exercise training on exercise performance, K^+ regulation, and skeletal muscle Na^+,K^+-ATPase function, content and (whole-muscle) isoform expression in humans

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	[^{3}H]ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
Aughey, Murphy, Clark, Garnham, Snow, Cameron-Smith, Hawley, McKenna[165]	Male cyclists or triathletes (n = 12) 65 mL/kg/min	2-3 sessions/wk for 3 wk 8 × 5-min cycling at 80% peak power output (1 min cycling at 100 W)	Peak power during incremental cycling to exhaustion ↑3%		3-O-MFPase activity ↑5%	0%	α_1, α_2, α_3: 0% β_1, β_2, β_3: 0%
Edge, Eynon, McKenna, Goodman, Harris, Bishop[234]	Recreationally-active women (n = 6) 46 mL/kg/min	3 sessions/wk for 5 wk 6-10 × 2 min at 140%-190% LT (1 min rest)	Power at VO_{2peak} ↑12%			↑22%	
Edge, Eynon, McKenna, Goodman, Harris, Bishop[234]	Recreationally-active women (n = 6) 46 mL/kg/min	3 sessions/wk for 5 wk 6-10 × 2 min at 140%-190% LT (3 min rest)	Power at VO_{2peak} ↑9%			↑26%	
Green, Chin, Ball-Burnett, Ranney[183]	Untrained men (n = 9) 48 mL/kg/min	6 sessions over 6 days 2-h cycling at 60%-65% VO_{2max}	Incremental cycling test to exhaustion (VO_{2max}) ↑7%	Venous plasma [K^+] ↓5%		↑14%	
Green, Dahly, Shoemaker, Goreham, Bombardier, Ball-Burnett[217]	Healthy and untrained subjects (n = 7), sex n/a 44.4 mL/kg/min	5-6 sessions/wk for 11 wk 2-h cycling at 68% VO_{2max}	Incremental cycling test to exhaustion (VO_{2max}) ↑15%			↑22%	
Green, Barr, Fowles, Sandiford, Ouyang[184]	Untrained men (n = 7) 46 mL/kg/min	6 sessions over 6 days 2-h cycling at 60%-65% VO_{2max}			3-O-MFPase activity ↑41%	↑9%	α_1: ↑16% α_2: ↑9% β_1: ↑39%
Green, Duhamel, Stewart, Tupling, Ouyang[185]	Recreationally-active men (n = 6) and women (n = 6) 45 mL/kg/min	3 sessions over 3 days 2 h cycling at 60% VO_{2max}			3-O-MFPase activity ↑34%	↑12%	α_1: ↑46% α_2: ↑42% α_3: ↑31% β_1: ↑19%, β_2: ↑28% β_3::↑20%
Madsen, Franch, Clausen[235]	Male runners (n = 42) 55 mL/kg/min	3 sessions/wk for 6 wk 25 min running at 93% HRmax	Running at pre-train 86% $VO_{2max}$$dummy$to exhaustion (TTE) ↑75%			↑15%	

(Continues)

TABLE 2 (Continued)

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	[^{3}H]ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
Benziane, Widegren, Pirkmajer, Henriksson, Stepto, Chibalin[182]	Healthy men (n = 8) 61 mL/kg/min	4 sessions over 10 days 45-90 min cycling at 75% $VO_{2max}$$dummy$and 6 × 5 min cycling at 90%-100% $VO_{2max}$$dummy$with 2 min cycling <40% $VO_{2max}$$dummy $between bouts on alternate days		Venous plasma [K+] ↓5%			α_1: ↑113% α_2: ↑49% α_3: 0% β_1: ↑27%, β_2: 0%
Evertsen, Medbo, Jebens, Nicolaysen[236]	Male (n = 11) and female (n = 9) cross country skiers 73 and 58 mL/kg/min	7 sessions/wk for 20 wk Running, roller- or cross-country skiing at moderate (40 min to 3 h at 60%-70% VO_{2max}) (86%) and high intensity (40 s to 7 min at 80%-90% 2max for 10 min to 2 h) (14%)	20-min running time trial ↑2%			↑16%	
Evertsen, Medbo, Jebens, Nicolaysen[236]	Male (n = 11) and female (n = 9) cross country skiers 73 and 58 mL/kg/min	7 sessions/wk for 20 wk Running, roller- or cross-country skiing at 80%-90% VO_{2max} (10 min to 2 h) or 40 s to 7 min at similar intensity (83%) or at 60%-70% VO_{2max} (17%)	20-min running time trial ↑4%			↑16%	
Green, MacDougall, Tarnopolsky, Melissa[110]	Healthy men (n = 9) VO_{2max} n/a	3 sessions/wk for 8 wk 30-min one-legged cycling at 75%-87% peak power output (first 6 wk) and 5 × 3-min cycling at 100% pre-train peak power output (final 2 wk)	Unilateral cycling to fatigue at 95% VO_{2max} ↑400%			↑14%	
Green, MacDougall, Tarnopolsky, Melissa[110]	Healthy men (n = 9) VO_{2max} n/a	3 sessions/wk for 8 wk 30-min one-legged cycling at 75%-87% peak power output (first 6 wk) and 5 × 3-min cycling at 100% pre-train peak power output (final 2 wk) in normobaric systemic hypoxia	Unilateral cycling to fatigue at 95% VO_{2max} ↑510%			↑14%	

(Continues)

TABLE 2 (Continued)

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	[^{3}H]ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
Bangsbo, Gunnarsson, Wendell, Nybo, Thomassen[237]	Endurance-trained men (n = 17) 63 mL/kg/min	2-3 sessions/wk for 6-9 wks Addition of sessions of 8-12 × 30-s at 95% maximal speed (3 min rest)	Exhaustive supramaximal treadmill run at 130% VO_{2peak} ↑36%	Venous plasma [K+] ↓6%			α_1: 0%, α_2: ↑68% β_1: 0%
Gunnarsson, Christensen, Holse, Christiansen, Bangsbo[200]	Danish 2nd Division male soccer players (n = 16) 61 mL/kg/min	1 additional session/wk for 5 wk Addition of a session of 6-9 × 30-s runs at 90%-95% maximal intensity (3 min)	incremental running time to exhaustion ↑11%				α_1: 0%, α_2: 0% β_1: ↓13%
Harmer, Ruell, McKenna, Chisholm, Hunter, Thom, Morris, Flack[12]	type-1 diabetic men (n = 5) and women (n = 3) 3300 mL/min	3 sessions/wk for 7 wk 4-10 × 30-s 'all-out' cycling sprints, 3-4 min rest	Incremental cycling test to exhaustion (VO_{2max} and peak power) ↑11%	Venous plasma [K+] ↓7%		↑8%	
Harmer, Ruell, McKenna, Chisholm, Hunter, Thom, Morris, Flack[12]	Healthy men (n = 4) and women (n = 3) 3170 mL/min	3 sessions/wk for 7 wk 4-10 × 30-s 'all-out' cycling sprints, 3-4 min rest	Incremental cycling test to exhaustion (VO_{2max} and peak power) ↑11%	Venous plasma [K+] ↓7%		↑8%	
Iaia, Thomassen, Kolding, Gunnarsson, Wendell, Rostgaard, Nordsborg, Krustrup, Nybo, Hellsten, Bangsbo[198]	Male runners (n = 8) 56 mL/kg/min	3-4 sessions/wk for 4 wk 8-12 × 30-s running bouts at 90%-95% of the max. speed attained during a 30-s 'all-out' run (3 min rest bt sprints)	running to fatigue at 130% VO_{2max} and incremental test to exhaustion ↑5%	Venous plasma [K+] ↓8%			α_1: ↑29% α_2: ↑16% β_1: 0%
McKenna, Schmidt, Hargreaves, Cameron, Skinner, Kjeldsen[238]	Healthy, untrained men (n = 6) 51 mL/kg/min	3 sessions/wk for 7 wk 4-10 × 30-s maximal-intensity cycling sprints (2.5-4 min rest)	4 maximal 30-s sprints ↑6%	Venous plasma [K+] ↓19%		↑16%	
Mohr, Krustrup, Nielsen, Nybo, Rasmussen, Juel, Bangsbo[186]	Healthy men (n = 7) 49 mL/kg/min	3-6 sessions/wk for 8 wk 8 × 30-s runs at 130% VO_{2max}, 1.5 min rest	incremental exercise to exhaustion ↑15%	Venous plasma [K+] ↓0%			α_1: 0% α_2: ↑68% β_1: ↑31%
Dela, Holten, Juel[204]	Healthy subjects (n = 7) VO_{2max} n/a	3 sessions/wk for 6 wk 3-4 sets of 10-12 reps at 50%-80% 1RM, 1.5-2 min rest	Maximal leg press and knee-extensor force ↑77% and ↑29%				α_1: ↑37% α_2: ↑22% β_1: ↑33%

(Continues)

TABLE 2 (Continued)

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	[^{3}H]ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
Dela, Holten, Juel[204]	Type-II diabetes patients (n = 10) VO_{2max} n/a	3 sessions/wk for 6 wk 3-4 sets of 10-12 reps at 50%-80% 1RM, 1.5-2 min rest	Maximal leg press and knee-extensor force ↑75% and ↑42%				α_1: ↑45% α_2: ↑41% β_1: ↑47%
Green, Dahly, Shoemaker, Goreham, Bombardier, Ball-Burnett[217]	Healthy and untrained subjects (n = 9), sex n/a 45 mL/kg/min	3 sessions/wk for 12 wk Resistance training: 3 × 6-8 1RM of squats, leg press, leg extensions	Incremental cycling test to exhaustion (VO_{2max}) ↑0%			↑16%	
Medbo, Jebens, Vikne, Refsnes, Gramvik[239]	Male athletes (n = 23) VO_{2max} n/a	1-3 sessions/wk for 12 wk 5 × 4 squats at 50% 1RM (concentric phase) and 110%-135% 1RM (eccentric phase)	1RM squat and Number of squats at 70% 1RM to exhaustion ↑7% and ↑29%			↑15%	
Perry, Wyckelsma, Murphy, Steward, Anderson, Levinger, Petersen, McKenna[23]	Sedentary, healthy men (n = 4) and women (n = 2) 46 mL/kg/min	3 sessions/wk for 4 wk 3-4 sets of 8-12 reps of leg-press, knee-extension, hamstring curls, and calf raises at 65%-70% 1RM (1 min rest)	Time to fatigue cycling at 85% leg VO2 peak ↑31%	Venous plasma [K+] and Δ[K+]/work ratio ↓0%		0%	α_1, α_2, α_3: 0% β_1, β_2, β_3: 0%
Skovgaard, Christensen, Larsen, Rostgaard Andersen, Thomassen, Bangsbo[240]	Endurance-trained male runners (n = 12) 59 mL/kg/min	4 sessions/wk for 8 wk Two sessions of 4-12 × 30-s 'all-out' (3 min rest) and two sessions of resistance training (3 sets of 8 reps at 15RM to 4 sets of 4 reps at 4 RM, squats, deadlift, leg press)	10-km run, 1500 m run, Yo-Yo IR2, VO_{2max} ↑4%, ↑6%, ↑44% and ↑0%				α_1, α_2: 0% β_1: 0%
Skovgaard, Almquist, Bangsbo[187]	Endurance-trained male and female runners (n = 11) 59 and 50 mL/kg/min	6 sessions/wk for 6 wk Maintained high-frequency SET (four sessions of 8-12 × 30-s 'all-out' runs, 3.5 min rest) and moderate-intensity running (two sessions of 30-60 min running at 60%-80% HRmax)	TTE during intense exercise ↑12%	Venous plasma [K+] ↓0%			α_1, α_2: 0% β_1: ↑39%

(Continues)

TABLE 2 (Continued)

Study	Subjects	Training	Performance	K^+ regulation (% decline vs. pre training)	Na^+,K^+-ATPase activity	[^{3}H]ouabain binding site content	Na^+,K^+-ATPase-isoform abundance
Skovgaard, Almquist, Bangsbo[187]	Endurance-trained male and female runners (n = 7) 59 and 50 mL/kg/min	4 sessions/wk for 12 wk Maintained low-frequency SET (two sessions of 10 × 30-s 'all-out' runs, 3.5 min rest) and moderate-intensity running (two sessions of 30-60 min running at 60%-80% HRmax)	TTE during intense exercise ↑16%	Venous plasma [K+] ↓14%			α_1, α_2: 0% β_1: ↑58%
Skovgaard, Almquist, Bangsbo[241]	Endurance-trained male runners (n = 8) 59 mL/kg/min	4 sessions/wk for 6 wk Two sessions of 4-12 × 30-s 'all-out' (3 min rest) and two sessions of running for 30-60 min at 60%-80% HRmax	Incremental running to exhaustion (VO_{2max}) ↑20%				α_1, α_2: 0% β_1: 0%
Thomassen, Christensen, Gunnarsson, Nybo, Bangsbo[199]	Elite soccer players (n = 18) 55 mL/kg/min	4.5 sessions/wk for 2 wk Small-sided games (8 × 2 min at 88% HRmax, 1 min rest) and 10-12 × 25-30-s 'all-out' runs and 16 × 40-60-s runs at 84% HRmax, 40-60-s rest	RSA, total sprint time, fastest sprint time, Yo-Yo IR2 ↑3%, ↑2%, ↑0%, and ↑0%				α_1: 0%, α_2: ↑15% β_1: 0%
Nielsen, Mohr, Klarskov, Kristensen, Krustrup, Juel, Bangsbo[7]	Healthy men (n = 6) 50 mL/kg/min	3-5 sessions/wk for 7 wk 15 × 1 min at 150% thigh VO_{2peak} (3 min rest)	Incremental exercise to exhaustion ↑28%	Muscle interstitial [K+] ↓27%			α_1: ↑29%, α_2: ↑15% β_1: 0%
Nielsen, Mohr, Klarskov, Kristensen, Krustrup, Juel, Bangsbo[7]	Healthy men (n = 6) 50 mL/kg/min	3-5 sessions/wk for 7 wk 15 × 1 min at 150% thigh VO_{2peak} (3 min rest)	Incremental exercise to exhaustion ↑28%	Leg K + release ↓0%			α_1: ↑29%, α_2: ↑15% β_1: 0%
Mohr, Krustrup, Nielsen, Nybo, Rasmussen, Juel, Bangsbo[186]	Healthy men (n = 6) 50 mL/kg/min	3-6 sessions/wk for 8 wk 15 × 1 min at 150% thigh VO_{2peak} (3 min rest)	Incremental exercise to exhaustion ↑28%	Venous plasma [K+] ↓0%			α_1: 0%, α_2: 0% β_1: ↑34%

Thirty human training studies included measurement of two or more of the following factors: Na^+,K^+-ATPase activity, content, whole-muscle isoform abundance, and K^+ regulation. Two studies were excluded as their main focus was on the effect of the environment (altitude exposure) rather than training ,[112,113] and one because training sessions were performed on a single day .[242] Searches were conducted on Pubmed, Science Direct, Google Scholar, and Web of Science using words such as: Na^+,K^+-ATPase, performance, training, human muscle. Manual searches were also performed using reference lists of published studies.

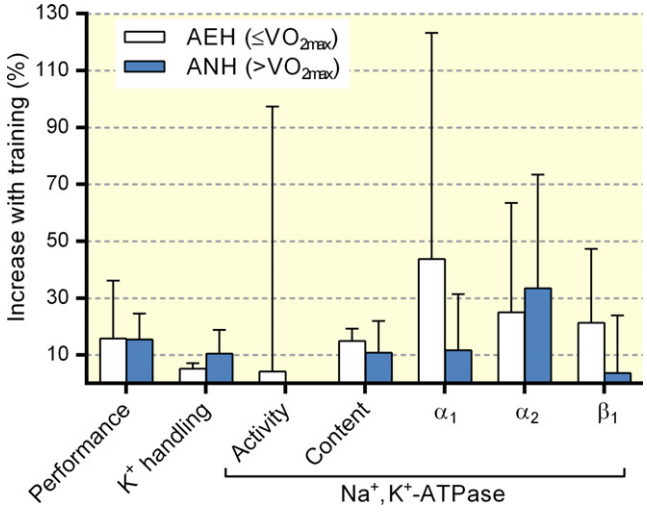

FIGURE 3 Effects of different types of exercise training on intense exercise performance, K^+ regulation (ie plasma blood K^+ concentration, thigh K^+ release, or muscle interstitial K^+ accumulation), and skeletal muscle Na^+,K^+-ATPase function, content, and isoform (α_1, α_2, β_1) abundance in humans. AEH = Training at intensities $\leq VO_{2max}$ (n = 13 interventions) ,[110,165,182–185,217,234–,236] ANH = Training at intensities $>VO_{2max}$ (n = 8 interventions).[7,12,186,198,200,237,238] Note that measurement of Na^+,K^+-ATPase activity was not included in studies on ANH training. Data are expressed as means +95% confidence intervals

(fibre-type-heterogeneous) samples in a substantial number of studies.[7,165,185–187] For example, three consecutive days of continuous training (2 hours cycling at 60% VO_{2max}) increased (12%) [^{3}H]-ouabain binding site content, but decreased (34%) 3-O-MFPase activity in recreationally-active subjects.[185] In similarly trained men, muscle abundance of Na^+,K^+-ATPase α_1 (29%) and α_2 (15%) increased and $[K^+]_{int}$ decreased (−27%), whereas K^+ release from exercising muscles remained unaltered after 7 weeks of intense interval training (15 × 1 minutes at 150% of leg VO_{2max}).[7] Further, 6-11 weeks of anaerobic training resulted in an increased β_1 abundance (39% to 58%), despite a higher (14%) venous blood $[K^+]$ during exercise in highly-trained runners.[187] The dissociation between exercise training-induced changes in Na^+,K^+-ATPase activity and protein abundance may, in part, be related to a lack of sensitivity of the 3-O-MFPase method to fluctuations in Na^+ affinity.[188] Such fluctuations are important to take into account if Na^+,K^+-ATPase activity is compared in skeletal muscle biopsies obtained from the same individual before and after a training period even at rest, because exercise training may increase basal FXYD1 phosphorylation [46,189] that in turn could raise Na^+,K^+-ATPase Na^+-affinity and thus function.[76] However, as highlighted in the following section of this review, limitations in protein analysis could also, at least partly, account for these dissociated outcomes.

Thus, among the published human studies to date, there is a consensus that exercise training, regardless of whether it is performed below/at or above the intensity eliciting VO_{2max}, is a powerful stimulus to enhance K^+ regulation, as well as Na^+,K^+-ATPase function, content, and isoform abundance. Furthermore, increases in these variables are often temporally associated with improvements in one or more aspects of physical performance following a period of intense training. However, training-induced alterations in blood $[K^+]$ or Na^+,K^+-ATPase activity were dissociated from those of Na^+,K^+-ATPase (isoform) expression in a substantial number of studies. Based on the above evidence from the training literature, it remains unclear what is the optimal training protocol (primarily aerobic vs. primarily anaerobic) for improving skeletal muscle K^+ regulation in humans. In the third part of this review dealing with blood-flow-restricted training, it is discussed how anaerobic (severe hypoxia and acidification) and aerobic (increased oxygenation) stimuli may be best utilized to promote improvements in muscle K^+ regulation in humans.

6 | FIBRE-TYPE-DEPENDENT REGULATION OF MUSCLE NA^+,K^+-ATPASE-ISOFORM ABUNDANCE BY DIFFERENT TYPES OF EXERCISE TRAINING IN HUMANS

Human skeletal muscle is a heterogeneous tissue consisting of fibres with distinct metabolic and ionic properties. Due to these differences, fibres can be defined according to their content of proteins with different functions. For example, fibres may be characterized by their content of myosin heavy chain (MHC) isoforms as type I, IIa, IIx, or hybrid if containing multiple MHC isoforms (eg I/IIa or IIa/IIx). In comparison to type II (fast-twitch) fibres, type I (slow-twitch) fibres have a slower rate of force development and SR Ca^{2+} release and uptake kinetics, altered glycogen utilization, possess more mitochondria, and are more fatigue resistant.[190–195] In animals, differences in the capacity for Na^+/K^+ handling among different skeletal muscles have also been observed, and this has been associated with a different expression of Na^+,K^+-ATPase isoforms between different fibre types.[41,78,196] In humans, α_2 abundance was found to be higher in type II compared to type I skeletal muscle fibres in recreationally-active men.[197] Furthermore, FXYD1 was more highly expressed in type I compared to type II muscle fibres in sedentary rats,[69,78] and in humans its phosphorylation state was rapidly increased in type II but not in type I muscle fibres after a single session of intense exercise.[197] Collectively, these studies suggest that expression of Na^+,K^+-ATPase isoforms and FXYD1 activation (by phosphorylation) may be altered by exercise in a fibre-type-dependent manner, which may significantly

impact K^+ regulation in the musculature. Nevertheless, in most human training studies, Na^+,K^+-ATPase-isoform abundance was quantified in fibre-type heterogeneous (whole-muscle) samples (Table 2), indicating that important changes in isoform levels could have been overlooked. Another concern is that protein abundance was quantified using fractionated samples in many previous studies.[7,184,198–201] This may be an issue, because a proportion of the protein being analysed may be inadvertently lost by fractionation.[202] In addition, some of the studies did not take into consideration blot linearity, making it impossible to know if protein bands were saturated and thus should be excluded from analysis.[203] Moreover, few studies validated their antibodies, for example by loading positive and/or negative control tissues. Limitations in methodology for protein quantification could thus be, at least, a contributory explanation for the dissociation between changes in expression of Na^+,K^+-ATPase isoforms in whole muscle samples and those of the capacity for K^+ regulation observed in the literature (Table 2), as highlighted in the previous section. With the use of improved methodology (ie no fractionation, normalization to a standard curve, and for some antibody validation), a number of recent human studies have investigated the effects of different types of exercise training on the expression of Na^+,K^+-ATPase isoforms in type I and II skeletal muscle fibres. The results from these studies are summarized in Table 3 and will be discussed in the following.

6.1 | α-isoform abundance

In one of our recent studies, 6 weeks of sprint-interval training increased (210% to 330%) α_1 abundance in both type I and II fibres in the skeletal muscle of recreationally-active men.[45] In agreement, 4 weeks of sprint-interval training resulted in an elevated (29%) α_1 abundance in whole-muscle samples from similarly trained subjects.[198] In contrast, in two other studies, interval-endurance training, characterized by a high rate of aerobic energy consumption, was without effect on α_1 abundance in type I and II fibres.[43,46] However, performing the same type of training with reduced muscle blood flow, thereby increasing rate of anaerobic ATP production, caused a ~ 50% higher abundance of α_1 in both fibre types.[46] Furthermore, resistance training, characterized by repeated, near-maximal efforts interspersed by several minutes of rest, was found to increase (79%) α_1 abundance in type II fibres [23] and in whole-muscle samples [204] in sedentary individuals. Collectively, these results support that training above the intensity eliciting VO_{2max}, and thus the degree of metabolic and ionic stress in exercising muscles,[91] is important for training-induced increases in α_1-isoform abundance in both human muscle fibre types. This is consistent with the observation made in the first part of this review that drastic perturbations in both metabolic and ionic homeostasis are likely essential signals underlying elevated Na^+,K^+-ATPase expression. Moreover, the duration of intense exercise bouts, and thus the time spent with perturbed ion homeostasis, could also be decisive. For example, 4-8 weeks of sprint-interval training with a shorter sprint duration (4 to 6 seconds) compared to the above studies (30 seconds) had no effect on α_1 abundance in either type I or II fibres [42] or in whole-muscle homogenates [186] in recreationally-active humans. Together, this evidence supports that repeated, near-maximal exercise bouts of substantial duration (at least 30 seconds) punctuated by several minutes of rest is an effective training approach to increase α_1 abundance in both muscle fibre types in humans.

Exercise training comprising a high exercise volume has been demonstrated in several studies to be an effective stimulus to increase α_2 abundance in the skeletal muscle of both untrained[7,182,184] and recreationally active humans.[185,186] Accordingly, a large training volume appears to be required to elicit changes in muscle α_2 abundance at the fibre-type level. For example, an increase in α_2 abundance in type II fibres (30%) was observed after 576 minutes of moderate-intensity interval training performed over 12 weeks,[43] whereas 324 minutes of similar training over 6 weeks was insufficient to alter α_2 abundance in type I and II fibres.[46] Furthermore, no significant change in α_2 abundance was detected in both fibre types after a period of sprint-interval training with a low (45 minutes) exercise volume.[45] In addition, whole-muscle α_2 abundance was elevated after six,[204] but not 4 weeks [23] of resistance training in untrained subjects. However, a training-induced increase (76%) in α_2 abundance was evident in type I fibres in the latter study,[23] indicating 6 weeks of sprint-interval training is sufficient to invoke fibre type-specific changes in α_2 abundance. In contrast, in another study, 6 weeks of sprint-interval training did not result in significant increases in α_2 abundance in any fibre type. However, in the latter study, a quantitatively higher α_2 abundance was observed in around three quarters of type I and II fibres, indicating a small sample size and great inter-subject variability prevented a statistically significant result (ie a statistical type-II error). Moreover, a non-significant increase (30%) in α_2 abundance was evident in type-II fibres after training with reduced muscle blood flow.[46] This is in line with the concept that a greater proportion of type-II fibres must be recruited to sustain power output during continuous exercise when oxygen supply to skeletal muscle is compromised.[205] Together, above findings indicate that training volume and relative exercise intensity are important determinants of the fibre type-dependent regulation of α_2 abundance in human skeletal muscle. In keeping with this, the demand for Na^+/K^+ transport invoked by training on each fibre type could be influential,

TABLE 3 Fibre type-specific changes in Na^+,K^+-ATPase-isoform abundance with exercise training in human skeletal muscle

Study	Subjects	Training			Exercise performance	Measurement of K^+ handling	Whole-muscle Na^+,K^+-ATPase content	Na^+,K^+-ATPase-isoform abundance	
		Frequency and duration	Protocol	Type				Type I fibres	Type II fibres
Wyckelsma, McKenna, Serpiello, Lamboley, Aughey, Stepto, Bishop, Murphy[42]	Recreationally active men (n = 4) and women (n = 5) 42 mL/kg/min	3 sessions/wk for 4 wk	Three sets of 5 × 4-s all-out running sprints separated by 20 s of rest, with 4.5 min of rest between sets	Sprint-interval	n/a	n/a	n/a	$\alpha_1\ \alpha_2\ \alpha_3 \leftrightarrow$ $\beta_1\ \beta_2\ \beta_3 \leftrightarrow$	$\beta_1 \uparrow 33\%$ $\alpha_1\ \alpha_2\ \alpha_3 \leftrightarrow$ $\beta_2\ \beta_3 \leftrightarrow$
Perry, Wyckelsma, Murphy, Steward, Anderson, Levinger, Petersen, McKenna[23]	Sedentary, healthy men (n = 4) and women (n = 2) 46 mL/kg/min	3 sessions/wk for 4 wk	Three to four sets of 8-12 repetitions at 65%-70% 1RM, with 1 min rest between sets (Leg-press, knee-extension, hamstring curl, and calf raise)	Resistance	Cycling time to exhaustion at 85% leg VO_{2max} ↑31%	Venous plasma $[K^+] \leftrightarrow$ $\Delta[K^+]$/work ratio ↔	[^{3}H]ouabain binding ↔ $\alpha_1\ \alpha_2\ \alpha_3 \leftrightarrow$ $\beta_1\ \beta_2\ \beta_3 \leftrightarrow$	$\alpha_2 \uparrow 76\%$ $\alpha_3 \uparrow 143\%$ $\alpha_1\ \beta_1\ \beta_2\ \beta_3 \leftrightarrow$	$\alpha_1 \uparrow 79\%$ $\beta_1 \uparrow 35\%$ $\alpha_2\ \alpha_3\ \beta_2$ $\beta_3 \leftrightarrow$
Wyckelsma, Levinger, Murphy, Petersen, Perry, Hedges, Anderson, McKenna[43]	Recreationally active older adults (69 years) men (n = 6) and women (n = 2)	3 sessions/wk for 12 wk	4 × 4-min cycling bouts at 90%-95% HR_{peak} separated by 4 min recovery at 50%-60% HR_{peak}	Aerobic-interval	Aerobic peak power output during cycling ↑ 25%	Peak venous plasma $[K^+] \uparrow$ ($P = 0.07$) $\Delta[K^+]$/work ratio ↔	[^{3}H]ouabain binding ↑ 11% $\alpha_1\ \alpha_2\ \beta_1 \leftrightarrow$	$\alpha_1\ \alpha_2\ \beta_1 \leftrightarrow$	$\alpha_2 \uparrow 30\%$ $\alpha_1\ \beta_1 \leftrightarrow$
Christiansen D, Eibye KH, Rasmussen V, Voldbye HM, Thomassen M, Nyberg M, Gunnarsson TGP, Skovgaard C, Lindskrog MS, Bishop DJ, Hostrup M, Bangsbo J[46]	Recreationally active men (n = 10) 50 mL/kg/min	3 sessions/wk for 6 wk	Three series of 3 × 2-min cycling bouts at 60%-80% peak aerobic power (1 and 2 min rest between bouts and series)	Aerobic-interval	Aerobic peak power output during cycling ↑ 11%	Arterial +Venous plasma $[K^+] \downarrow$ a-v-K^+ difference ↔ thigh K^+ release ↔	n/a	$\beta_1 \downarrow 18\%$ $\alpha_1\ \alpha_2 \leftrightarrow$ FXYD1 ↔	$\alpha_1\ \alpha_2\ \beta_1 \leftrightarrow$ FXYD1 ↔

(Continues)

TABLE 3 (Continued)

Study	Subjects	Training			Exercise performance	Measurement of K^+ handling	Whole-muscle Na^+,K^+-ATPase content	Na^+,K^+-ATPase-isoform abundance	
		Frequency and duration	Protocol	Type				Type I fibres	Type II fibres
Christiansen D, Eibye KH, Rasmussen V, Voldbye HM, Thomassen M, Nyberg M, Gunnarsson TGP, Skovgaard C, Lindskrog MS, Bishop DJ, Hostrup M, Bangsbo J[46]	Recreationally active men (n = 10) 50 mL/kg/min	3 sessions/wk for 6 wk	Three series of 3 × 2-min cycling bouts at 60%-80% peak aerobic power (1 and 2 min rest between bouts and series) + blood flow restriction	Aerobic-interval +blood flow restriction	Aerobic peak power output during cycling ↑ 23%	Arterial +Venous plasma $[K^+]$ ↓ a-v-K^+ difference ↓ thigh K^+ release ↓	n/a	α_1 ↑ 46% ($P = 0.075$) FXYD1 ↑ 108% α_2 β_1 ↔	α_1 ↑51% FXYD1 ↑ 60% α_2 β_1 ↔
Christiansen, Bishop, Broatch, Bangsbo, McKenna, Murphy[45]	Recreationally active men (n = 7) 45 mL/kg/min	3 sessions/wk for 6 wk	4-6 × 30-s all-out cycling sprints separated by 4 min of rest	Sprint-interval	Aerobic peak power output during cycling ↑ 6% Incremental 2-km cycling time trial ↑ 3.3% 20-km cycling time trial ↔	n/a	n/a	α_1 ↑ 210% β_3 ↑ 300% FXYD1 ↓33% α_2 α_3 β_1 ↔	α_1 ↑ 270% β_1 ↑ 44% β_3 ↑ 410% α_2 α_3 FXYD1 ↔
Christiansen, Bishop, Broatch, Bangsbo, McKenna, Murphy[45]	Recreationally active men (n = 5) 45 mL/kg/min	3 sessions/wk for 6 wk	4-6 × 30-s all-out cycling sprints separated by 4 min of rest + Cold-water immersion	Sprint-interval +Cold-water immersion	Aerobic peak power output during cycling ↑ 6% 2-km cycling time trial ↑ 2.4% 20-km cycling time trial ↔	n/a	n/a	α_1 ↑ 330% β_3 ↑ 540% FXYD1 ↓33% α_2 α_3 β_1 ↔	α_1 ↑ 260% β_1 ↑ 44% β_3 ↑ 400% α_2 α_3 ↔ FXYD1 ↔

consistent with the finding in vitro that the major role of α_2 in skeletal muscle is to assure that contraction-stimulated increases in demand for Na^+/K^+ transport are met.[74]

The abundance of the α_3 isoform remained unchanged with both interval-endurance [46] and sprint-interval training [45] in both muscle fibre types in humans. This is consistent with observations in whole-muscle samples of unchanged α_3 abundance after 3 weeks of interval cycling with a high aerobic component (8 × 5-minute at 85% VO_{2max}) in well-trained men.[165] Although only semi-quantitative, western blots for α_3 indicate that this isoform may be lowly expressed at the protein level in human skeletal muscle, which is in accordance with the low α_3 mRNA expression detected previously in the same tissue.[165] These observations downplay the functional importance of α_3 for the muscle's contractile function in humans, which could be one explanation for the lack of change in α_3 abundance in both fibre types with the different types of training studied. In contrast to these observations, α_3 abundance increased (31%) in whole-muscle samples after three consecutive days with 2 hours of cycling at 60% VO_{2max} per day,[185] indicating exercise training, in some cases, can increase α_3 abundance in human muscle, although the physiological reason(s) for the latter finding is unclear.

6.2 | β-isoform abundance

In one human study, 4 weeks of sprint-interval training increased the abundance of β_1 in type II, but not in type I fibres (identified in individual fibre segments).[42] In agreement, we have recently found that β_1 abundance was selectively increased (44%) in type II fibres after 6 weeks of sprint-interval training in humans.[45] The α-isoform abundance was also raised in the same fibre type in the latter study, suggesting that intense training requires a high functional capacity of the Na^+,K^+-ATPase in type II fibres. In support, in rat gastrocnemius muscle, higher Na^+,K^+-ATPase hydrolytic activity was reported in membrane vesicles with a reduced (50%) molar α_2/β_1 ratio caused by higher β_1 content, relative to vesicles with a greater ratio (1.0).[67] In contrast to the selective increase in type-II fibre β_1 abundance by sprint-interval training, β_1 abundance was reported to decrease by 18% in type I fibres after 6 weeks of interval-endurance training.[46] While the decrease is likely unrelated to method variation, because technical variability in measurement was low (<12%), it might be explained by a possible detraining effect given that the subjects in the latter study was used to train at a substantially higher intensity. Accordingly, increasing relative intensity by reducing muscle blood flow during training attenuated the decline in type-I fibre β_1 abundance with the training period.[46] Collectively, these results suggest that β_1 is regulated in a fibre type-specific manner to some types of exercise training (and detraining) in human skeletal muscle. This regulation appears to be dependent on relative training intensity and thus the demands imposed by training on transmembrane Na^+/K^+ transport in the different muscle fibre types.

Findings from our lab indicate that β_2 is lowly expressed in human vastus lateralis muscle,[45] suggesting even large changes in β_2 abundance in one type or both fibre types with training may be of small relevance for the skeletal muscle ion transport function in humans. Accordingly, neither sprint-interval [42] nor resistance training [23] resulted in altered β_2 abundance in type I or II fibres in humans. However, a higher (27%) β_2 abundance has been observed in type II compared to type I fibres identified in individual fibre segments from human skeletal muscle [42]; a finding we have recently confirmed in separate pools of type I and II fibres using a modified method for fibre-type-specific protein analysis.[45] These results highlight that β_2, although not altered in any fibre type by several types of intense training (Table 3), is expressed in a fibre type-specific manner in human skeletal muscle. Whilst it might be suggested that this pattern of expression might be functionally relevant, supported by the observation in rat skeletal muscle that the K_m for Na^+ of α/β_2 heterodimers (7.5-13 mmol/L) is higher than the corresponding K_m for α/β_1 complexes (4-5.5 mmol/L),[196] this remains to be elucidated in humans.

Na^+,K^+-ATPase β_3 abundance has been shown to be elevated in type I (onefold) and II (threefold) muscle fibres, and in whole-muscle homogenates (2.5-fold), with age in humans.[21,206] In rat skeletal muscles, a similar age-associated increase in β_3 abundance was reversed by 14 weeks of endurance training.[207] Thus, regular continuous muscle activity potently attenuates age-induced increases in β_3 abundance in human muscle. In contrast, sprint-interval training increased β_3 abundance by more than two fold in both human muscle fibre types.[45] Together, these findings underline that β_3 abundance is similarly regulated in type I and II muscle fibres in humans and that this regulation appears to be dependent on the type of muscle activity (or lack thereof) regularly undertaken. In one of the human studies, the increase in β_3 abundance with sprint-interval training occurred concomitant with an increase in α_1 abundance,[45] suggesting an enhanced potential for α_1/β_3 complex assembly in both fibre types after the training period. This supports that the β_3 isoform could take part in maintenance of resting membrane potential in both fibre types, in line with the ion transport function of the α_1 isoform.[74] However, this warrants further investigation.

6.3 | FXYD1 abundance

Previous human studies using whole-muscle samples reported no alterations in FXYD1 protein abundance

following10 days to 8 weeks of intense training.[113,182,199,208] In contrast, we have recently shown that 6 weeks of sprint-interval training decreased FXYD1 abundance by 33% in type I fibres.[45] FXYD1 abundance remained unchanged in type II fibres in the same study, highlighting FXYD1 abundance is regulated in a fibre type-dependent manner by intense training in human skeletal muscle. As FXYD1 may regulate Na^+,K^+-ATPase function in multiple ways (eg by control of oxidation [75] and Na^+ affinity[209]), this indicates that physiologically relevant adaptations could be overlooked by the use of whole-muscle homogenate for protein analyses. Other methodological steps should also be avoided, including sample fractionation, ie removal of an indefinite amount of protein.[202] In another human study, FXYD1 abundance was higher in both type I and II fibres after interval-endurance training with compared to without reduced muscle blood flow,[46] indicating relative exercise intensity is important for alterations in FXYD1 abundance at the fibre-type level with training in humans. The different regulation of FXYD1 abundance in type I fibres with training in the latter two studies is likely explained by differences in work-to-rest ratio, and/or training duration (approximately 45 vs. 324 minutes).

6.4 | Summary

In summary of the scarce number of published human studies on this topic, the abundance of Na^+,K^+-ATPase α_1 and β_3 appears to be similarly altered in type I and II muscle fibres by different types of training in humans. Furthermore, increases in both α_1, α_2, and β_1 abundance by exercise training are likely training-intensity-dependent, consistent with the role of both metabolic, ion and redox perturbations in regulating Na^+,K^+-ATPase expression. The abundance of α_2 may also be regulated according to training volume. The regulation of the α_2 isoform to different types of training appears to occur in a fibre-type dependent manner and this may be a result of the extent to which the different fibre types are recruited while training. Both the isoforms α_3 and β_2 may be lowly expressed in human skeletal muscle and the expression of α_3 hardly altered at the fibre type level by training in humans. The existing evidence suggests that changes in the abundance of α_3 and β_2 may be of little functional importance for the ion transport capacity of human skeletal muscle. Moreover, FXYD1 is upregulated in both muscle fibre types to certain types of intense training (ie blood-flow-restricted training), but not in response to other types (eg sprint-interval) in human skeletal muscle. The regulation of FXYD1 likely depends on training intensity, work:rest ratio, exercise duration, or a combination of these factors.

7 | IMPLICATIONS OF FIBRE TYPE-SPECIFIC ADAPTATIONS IN NA^+,K^+-ATPASE-ISOFORM EXPRESSION FOR K^+ REGULATION IN HUMANS: NOVEL INSIGHTS FROM BLOOD FLOW-RESTRICTED TRAINING

In most published studies that have examined training-induced effects on Na^+,K^+-ATPase abundance in type I and II muscle fibres in humans, measurement of K^+ regulation was either not included [42] or K^+ concentration was measured in venous plasma,[23,43] which poorly reflects K^+ homeostasis at the muscle level.[7,9] In a recent experiment, we assessed the effects of exercise training with and without blood flow restriction (BFR) on Na^+,K^+-ATPase-isoform abundance at the fibre-type level, together with measurement of net thigh K^+ release during isolated work with the quadriceps muscle.[46] A novel observation was that higher abundance of β_1 (33%) and FXYD1 (108%) in type I, and α_1 (51%) and FXYD1 (60%) in type II fibres occurred concomitant with a reduced net rate of thigh K^+ release during intense exercise after training with BFR. Furthermore, in the three subjects where α_2 abundance was determined, a large effect for an increase in α_2 was evident in both type I (38%, $d = 0.8$) and type II fibres (38%, $d = 0.7$) after the training period with BFR. In contrast, simultaneous training of the contralateral leg without BFR neither increased isoform levels nor altered thigh K^+ release.[46] Based on these findings, the authors of the latter study concluded that, adaptations in the expression of Na^+,K^+-ATPase isoforms at the fibre-type level appear to be important for muscle K^+ regulation during exercise in humans.[35] Moreover, consistent with previous observations in humans,[6,8] enhancement of muscle K^+ regulation in the study highlighted above was temporally associated with an improved exercise tolerance after training with BFR (11% greater vs. control leg),[46] providing support for a positive association between locomotor muscle K^+ regulation and exercise performance.

8 | REGULATION OF NA^+,K^+-ATPASE-ISOFORM mRNA IN HUMAN SKELETAL MUSCLE BY A SINGLE EXERCISE SESSION: RELATION TO PROTEIN CHANGES WITH EXERCISE TRAINING

Changes in steady-state protein abundance may often be partly determined by variance in mRNA levels.[210] A single exercise session increases the mRNA of a growing number of genes,[211–213] and these increases may often occur prior to upregulation of protein content in human skeletal

muscle.[214] Thus, measurement of mRNA responses to a single exercise session can often provide valuable insights into the potential of a given training strategy to modulate abundance of proteins and ultimately their function. However, this assumption should be carefully regarded, because of the complex process that underpins upregulation of protein levels. Increases in protein are not exclusively a result of elevated mRNA availability, but also depends on efficient mRNA translation and protein stabilization (synthesis relative to degradation). Further complicating interpretation, mRNA availability is rapidly altered according to mRNA transcription, alternative splicing, synthesis, decay, and translation.[215]

The cross-sectional data summarized in Table 4 indicate that Na^+,K^+-ATPase isoforms ($\alpha_{1\text{-}3}$ and $\beta_{1\text{-}3}$) may be partly regulated at the mRNA level by exercise in humans. By comparing the responses in mRNA expression to a single exercise session (Table 4) with corresponding changes in protein content to a period of exercise training (Table 2), it could be argued that effects of an exercise session on Na^+, K^+-ATPase mRNA transcript levels reflect changes in corresponding isoform protein abundance after a period of the same type of training. For example, an increase (3 fold) in muscle α_1 mRNA was observed in recovery from a single session consisting of fifteen 1-minute exercise bouts at 150% of leg VO_{2max} separated by 3 minutes of rest,[164] whereas the same training protocol performed 3-5 times per week for 7 weeks resulted in an elevated (29%) α_1 protein abundance in what appears to be the same individuals.[7] Furthermore, increases in α_1 (2 fold),[216] α_2 (1.8 fold), and α_3 mRNA (3.3 fold) [92] have been reported after a session of continuous aerobic exercise (45 to 55 minutes at 71% to 75% of VO_{2max}). In comparison, 6 days-11 weeks of regularly performing continuous aerobic training (2 hours at 60%-65% VO_{2max}) resulted in elevated α_1 (16%) and α_2 (9%) protein abundance and [^{3}H]-ouabain binding site content (9% to 14%).[183,184,217] Furthermore, an increase in [^{3}H]-ouabain binding (14%) was reported after several weeks of intense aerobic interval training with one leg (5 × 3-minute of unilateral cycling at 100% of pre-train aerobic peak power), whereas one session with the same type of exercise (5 × 2-5 minutes at 56 ± 5 W and 60 kick/min separated by 3 minutes of rest) elevated the mRNA levels of α_1 (3.8 fold) and α_2 (2.4 fold) in recreationally-active subjects. In another study, a session of sprint-interval exercise increased both α_1 and β_3 mRNA content, whereas 6 weeks of performing the same type of training caused an elevated protein abundance of these isoforms in muscle of recreationally-active men.[45] In addition, 6 weeks of interval-endurance training with BFR resulted in elevated FXYD1 protein abundance in both muscle fibre types. Accordingly, a single session comprising of the same type of training increased FXYD1 mRNA content in human skeletal muscle.[91] However, an association between mRNA and protein adaptations is not a universal finding. For example, no change in α_2 mRNA was observed after a session of intense interval exercise,[164] despite an increase in α_2 protein (15%) after 7 weeks of regularly performing the same type of exercise.[7] Similarly, a selective increase in α_2 mRNA after a single session of post-exercise cold-water immersion was observed, despite no change at the protein level with 6 weeks of training, where each training session was concluded with cold-water immersion.[45] These observations in human muscle are supported by a similar dissociation between responses of α_2 mRNA and protein to thyroid hormone in cultured skeletal muscle cells [218] and to sprint-interval training in rat EDL and soleus muscles.[172]

In summary, there are some indications that the accumulative effects of repeated exercise-induced changes in mRNA is an important determinant of net protein turnover of most Na^+,K^+-ATPase isoforms after several weeks of training. However, it is clear from the current evidence that variance in mRNA is likely one of several factors that control protein levels of these isoforms in human skeletal muscle following a period of exercise training; indicating altered mRNA to a single training session is not a solid marker of potential protein outcomes for these isoforms after several weeks of training.

9 | AN ALTERNATIVE APPROACH TO DESIGNING EXERCISE TRAINING INTERVENTIONS TO MAXIMIZE IMPROVEMENTS IN K^+ REGULATION

Based on the previous sections of this review, a key question that remains to be answered is what strategy should be chosen to most effectively enhance (ie within shortest time with maximal benefit) K^+ regulation and thus fatigue tolerance during intense exercise in humans. In recent years, we in our laboratory have focused on developing a training strategy to maximally stimulate the molecular stressors identified in cell culture and animal models (in vitro) to be prerequisite to beneficial adaptations specific to K^+ regulation, with the aim to maximize training adaptation. This alternative approach to optimizing training has with success been centered on the use of blood flow restriction (BFR) to manipulate blood perfusion of exercising muscles.[46,91] With emphasis on this training strategy, the aim of this section is to provide the reader with an alternative view on how training interventions may be designed to maximize skeletal muscle adaptation in humans.

Blood flow restriction (BFR) typically involves inflation of a pneumatic tourniquet (or cuff) around the most proximal portion of the limbs, thereby reducing muscle blood

TABLE 4 Literature summary of the effects of a single exercise session on mRNA content of Na^+, K^+-ATPase isoforms in human skeletal muscle

Study	Subjects	Exercise protocol	Metabolic changes	Fold-changes in mRNA content of Na^+,K^+-ATPase isoforms						
				α_1	α_2	α_3	β_1	β_2	β_3	FXYD1
Christiansen, Murphy, Bangsbo, Stathis, Bishop[91]	Recreationally active (trained) men (n = 8) 57 mL/kg/min	Three sets of 3 × 2 min running bouts at 105% lactate threshold (~12 km/h), with bouts separated by 1 min and sets by 5 min. Blood flow restriction during bouts: ~175 mmHg.	Peak muscle lactate: ~50 mmol/kg dw muscle Peak blood lactate: ~6.7 mmol/L Minimum blood pH: ~7.2 Peak blood K^+: ~5.1 mmol/L	3 h: →	3 h: ~1.6↑	3 h: →	3 h: →	3 h: →	3 h: →	3 h: ~2.7↑
Christiansen, Murphy, Bangsbo, Stathis, Bishop[91]	Recreationally active (trained) men (n = 8) 57 mL/kg/min	Three sets of 3 × 2 min running bouts at 105% lactate threshold (~12 km/h), with bouts separated by 1 min and sets by 5 min. Systemic hypoxia during bouts: ~3250 m altitude.	Peak muscle lactate: ~42 mmol/kg dw muscle Peak blood lactate: ~6.4 mmol/L Minimum blood pH: ~7.2 Peak blood K^+: ~5.1 mmol/L	3 h: →	3 h: ~1.6↑	3 h: →	3 h: ~1.7↑	3 h: →	3 h: →	3 h: →
Christiansen, Murphy, Bangsbo, Stathis, Bishop[91]	Recreationally active (trained) men (n = 8) 57 mL/kg/min	Three sets of 3 × 2 min running bouts at 105% lactate threshold (~12 km/h), with bouts separated by 1 min and sets by 5 min.	Peak muscle lactate: ~28 mmol/kg dw muscle Peak blood lactate: ~3.5 mmol/L Minimum blood pH: ~7.25 Peak blood K^+: ~5.0 mmol/L	3 h: →	3 h: →	3 h: →	3 h: →	3 h: →	3 h: →	3 h: →
Christiansen, Bishop, Broatch, Bangsbo, McKenna, Murphy[45]	Recreationally active men (n = 10) 45 mL/kg/min	Four 30-s 'all-out' cycling sprints separated by 4 min of rest.	n/a	0 h: → 3 h: ~2↑	0 h: → 3 h: →	0 h: → 3 h: →	0 h: → 3 h: →	0 h: → 3 h: ↓ to 0.7	0 h: → 3 h: ~2↑	0 h: → 3 h: →
Christiansen, Bishop, Broatch, Bangsbo, McKenna, Murphy[45]	Recreationally active men (n = 9) 45 mL/kg/min	Four 30-s 'all-out' cycling sprints separated by 4 min of rest with post-exercise cold-water immersion (15 min at 10°C).	n/a	0 h: → 3 h: ~2↑	0 h: ~2.1↑ 3 h: →	0 h: → 3 h: →	0 h: → 3 h: →	0 h: → 3 h: ↓ to 0.7	0 h: → 3 h: ~2.5↑	0 h: → 3 h: →
Nordsborg, Bangsbo, Pilegaard[164]	Healthy males (n = 6) *Trained leg* 50 mL/kg/min	15 × 1 min at 150% leg VO_{2peak} separated by 3 min of rest	n/a	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	n/a	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	n/a	n/a	n/a

(Continues)

TABLE 4 (Continued)

Study	Subjects	Exercise protocol	Metabolic changes	Fold-changes in mRNA content of Na^+,K^+-ATPase isoforms						
				α_1	α_2	α_3	β_1	β_2	β_3	FXYD1
Nordsborg, Thomassen, Lundby, Pilegaard, Bangsbo[243]	Recreationally active males (n = 8) VO_{2max} n/a	5 × 2-5 min at 56 ± 5 W and 60 kick/min separated by 3 min of rest	Blood lactate = 4.2 ± 2.2 mmol/L Epinephrine: 0.3 ± 0.1 nmol/L Norepinephrine: 0.8 ± 0.3 nmol/L	0 h: → 1 h: ~3.8↑ 3 h: ~3.5↑ 5 h: ~2.5↑	0 h: → 1 h: ~2.1↑ 3 h: ~2.4↑ 5 h: ~1.9↑	n/a	0 h: → 1 h: ~2.8↑ 3 h: ~2.7↑ 5 h: ~2.2↑	0 h: → 1 h: → 3 h: → 5 h: →	0 h: ~1.9↑ 1 h: ~2.2↑ 3 h: ~3.1↑ 5 h: ~2↑	n/a
Nordsborg, Thomassen, Lundby, Pilegaard, Bangsbo[243]	Recreationally active males (n = 8) VO_{2max} n/a	5 × 2-5 min at 56 ± 5 W and 60 kick/min separated by 3 min of rest	Blood lactate = 11.4 ± 4.2 mmol/L Epinephrine: 0.75 ± 0.45 nmol/L Norepinephrine: 7.5 ± 5.5 nmol/L	0 h: → 1 h: ~3.0↑ 3 h: ~3.8↑ 5 h: ~2.5↑	0 h: → 1 h: ~1.9↑ 3 h: ~1.9↑ 5 h: ~1.9↑	n/a	0 h: → 1 h: ~2↑ 3 h: ~2.1↑ 5 h: →	0 h: → 1 h: → 3 h: → 5 h: →	0 h: ~2.0↑ 1 h: ~2.0↑ 3 h: ~2.2↑ 5 h: ~2.0↑	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard [168]	Untrained (n = 8) 44 mL/kg/min	4 × 4 min at 83% ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 10.8 ± 0.5 mmol/L (arm vein)	1 + 3 h: ~2.0↑	1 + 3 h: →	n/a	1 + 3 h: ~2.1↑	n/a	n/a	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard [168]	Trained males (n = 10) 55 mL/kg/min	4 × 4 min at 85% ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 10.1 ± 0.4 mmol/L (arm vein)	1 + 3 h: ~2.0↑	1 + 3 h: →	n/a	1 + 3 h: ~1.7↑	n/a	n/a	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard[168]	Trained males (n = 6) 55 mL/kg/min	4 × 4 min at 70% ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 3.1 ± 0.7 mmol/L (arm vein)	1 + 3 h: ~1.4↑	1 + 3 h: →	n/a	1 + 3 h: →	n/a	n/a	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard[168]	Untrained (n = 6) 44 mL/kg/min	~3 h 20 min at 44% ± 1% VO_{2peak} (exhaustion)	n/a	1 h: ~3.0↑	1 h: ~1.8↑	n/a	1 h: →	n/a	n/a	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard[168]	Trained males (n = 6) 55 mL/kg/min	~3 h 45 min at 44% ± 1% VO_{2peak} (exhaustion)	n/a	1 h: →	1 h: →	n/a	1 h: →	n/a	n/a	n/a

(Continues)

TABLE 4 (Continued)

Study	Subjects	Exercise protocol	Metabolic changes	Fold-changes in mRNA content of Na^+,K^+-ATPase isoforms						
				α_1	α_2	α_3	β_1	β_2	β_3	FXYD1
Petersen, Murphy, Snow, Leppik, Aughey, Garnham, Cameron-Smith, McKenna[166]	Healthy males (n = 8) and females (n = 7) 51 mL/kg/min	5 min 52 s ± 4 min 27 s at 40% of total work (exhaustion)	Plasma volume: ~3.3%-12.3% ↓ Plasma [K^+]: ↑ from 3.9 (rest) to 4.3 (exercise peak) mmol/L	0 h: ~1.5↑ Mean of 0 + 3+24 h: ~1.5↑	0 h: ~2.5↑ Mean of 0 + 3+24 h: ~3.3↑	0 h: ~2.4↑ Mean of 0 + 3+24 h: ~1.4↑	0 h: → Mean of 0 + 3+24 h: ~1.1↑	0 h: ~1.7↑ Mean of 0 + 3+24 h: ~2.0↑	0 h: → Mean of 0 + 3+24 h: ~1.0↑	n/a
Nordsborg, Bangsbo, Pilegaard[164]	Healthy males (n = 6) *Trained leg* 50 mL/kg/min	15 × 1 min at 150% leg VO_{2peak} separated by 3 min of rest	n/a	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	n/a	0 h: → 1 h: → 3 h: → 5 h: → 24 h: →	n/a	n/a	n/a
Nordsborg, Thomassen, Lundby, Pilegaard, Bangsbo[243]	Recreationally active males (n = 8) VO_{2max} n/a	5 × 2-5 min at 56 ± 5 W and 60 kick/min separated by 3 min of rest	Blood lactate = 4.2 ± 2.2 mmol/L Epinephrine: 0.3 ± 0.1 nmol/L Norepinephrine: 0.8 ± 0.3 nmol/L	0 h: → 1 h: ~3.8↑ 3 h: ~3.5↑ 5 h: ~2.5↑	0 h: → 1 h: ~2.1↑ 3 h: ~2.4↑ 5 h: ~1.9↑	n/a	0 h: → 1 h: ~2.8↑ 3 h: ~2.7↑ 5 h: ~2.2↑	0 h: → 1 h: → 3 h: → 5 h: →	0 h: ~1.9↑ 1 h: ~2.2↑ 3 h: ~3.1↑ 5 h: ~2↑	n/a
Nordsborg, Thomassen, Lundby, Pilegaard, Bangsbo[243]	Recreationally active males (n = 8) VO_{2max} n/a	5 × 2-5 min at 56 ± 5 W and 60 kick/min separated by 3 min of rest	Blood lactate = 11.4 ± 4.2 mmol/L Epinephrine: 0.75 ± 0.45 nmol/L Norepinephrine: 7.5 ± 5.5 nmol/L	0 h: → 1 h: ~3.0↑ 3 h: ~3.8↑ 5 h: ~2.5↑	0 h: → 1 h: ~1.9↑ 3 h: ~1.9↑ 5 h: ~1.9↑	n/a	0 h: → 1 h: ~2↑ 3 h: ~2.1↑ 5 h: →	0 h: → 1 h: → 3 h: → 5 h: →	0 h: ~2.0↑ 1 h: ~2.0↑ 3 h: ~2.2↑ 5 h: ~2.0↑	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard[168]	Untrained (n = 8) 44 mL/kg/min	4 × 4 min at 83% ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 10.8 ± 0.5 mmol/L (arm vein)	1 + 3 h: ~2.0↑	1 + 3 h: →	n/a	1 + 3 h: ~2.1↑	n/a	n/a	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard[168]	Trained males (n = 10) 55 mL/kg/min	4 × 4 min at 85% ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 10.1 ± 0.4 mmol/L (arm vein)	1 + 3 h: ~2.0↑	1 + 3 h: →	n/a	1 + 3 h: ~1.7↑	n/a	n/a	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard[168]	Trained males (n = 6) 55 mL/kg/min	4 × 4 min at 70% ± 2% VO_{2peak} separated by 3 min of rest	Plasma lactate = 3.1 ± 0.7 mmol/L (arm vein)	1 + 3 h: ~1.4↑	1 + 3 h: →	n/a	1 + 3 h: →	n/a	n/a	n/a

(Continues)

TABLE 4 (Continued)

Study	Subjects	Exercise protocol	Metabolic changes	Fold-changes in mRNA content of Na^+,K^+-ATPase isoforms						FXYD1
				α_1	α_2	α_3	β_1	β_2	β_3	
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard[168]	Untrained (n = 6) 44 mL/kg/min	~3 h 20 min at 44% ± 1% VO_{2peak} (exhaustion)	n/a	1 h: ~3.0↑	1 h: ~1.8↑	n/a	1 h: →	n/a	n/a	n/a
Nordsborg, Kusuhara, Hellsten, Lyngby, Lundby, Madsen, Pilegaard[168]	Trained males (n = 6) 55 mL/kg/min	~3 h 45 min at 44% ± 1% VO_{2peak} (exhaustion)	n/a	1 h: →	1 h: →	n/a	1 h: →	n/a	n/a	n/a
Petersen, Murphy, Snow, Leppik, Aughey, Garnham, Cameron-Smith, McKenna[166]	Healthy males (n = 8) and females (n = 7) 51 mL/kg/min	5 min 52 s ± 4 min 27 s at 40% of total work (exhaustion)	Plasma volume: ~3.3%-12.3% ↓ Plasma $[K^+]$: ↑ from 3.9 (rest) to 4.3 (exercise peak) mmol/L	0 h: ~1.5↑ Mean of 0 + 3+24 h: ~1.5↑	0 h: ~2.5↑ Mean of 0 + 3+24 h: ~3.3↑	0 h: ~2.4↑ Mean of 0 + 3+24 h: ~1.4↑	0 h: → Mean of 0 + 3+24 h: ~1.1↑	0 h: ~1.7↑ Mean of 0 + 3+24 h: ~2.0↑	0 h: → Mean of 0 + 3+24 h: ~1.0↑	n/a

↓ = decrease; ↑ increase; → no change. Fold-changes are relative to baseline (ie rest and/or before exercise).

perfusion (Figure 4). This strategy has been applied during various types of exercise, including walking, cycling, running, and resistance training.[91,219–221] Superimposition of BFR during exercise leads to premature fatigue.[222] Although evidence in humans is lacking, this appears to relate, at least partly, to malfunction of ion channels and transport systems.[35,86,126] For example, inactivation of the Na^+-K^+-ATPase may occur earlier during exercise with than without BFR due, in part, to inhibition of its primary energy pathway (anaerobic glycolysis), promoted in part by intramuscular acidification.[35] BFR exercise may also result in the Na^+-K^+-ATPase becoming less active by increasing the formation of ROS and oxidative damage.[86,91,105] Oxidative inhibition of Na^+-K^+-ATPases, and consequently a decline in muscle fibre Na^+-K^+-ATPase activity, leads to an increase in $[Na^+]_i$ and a concomitant rise in $[Ca^{2+}]_{cyt}$ due in part to a decrease in the driving force for Ca^{2+} extrusion.[223,224] Accumulation of $[Ca^{2+}]_{cyt}$ may also be augmented by ROS formation due, in part, to their capability to inhibit the sarcoplasmic reticulum calcium ATPase (SERCA).[126] Thus, both $[K^+]$, $[Na^+]_i$ and $[Ca^{2+}]_{cyt}$ could be amplified by exercising with BFR. A rise in the inward osmotic pressure gradient accompanying BFR, forcing fluid to accumulate in the intracellular compartment, may further magnify the ionic perturbations during exercise, for example, by increasing the intracellular dilution space for K^+.[54] In humans, post-exercise muscle oxygenation, a key regulator of Na^+-K^+-ATPase expression in vitro,[96,98,99] has been shown to be augmented by exercising with BFR.[225,226] In summary, several key stressors involved in enhancing K^+ regulation, which were identified earlier in this review, could be augmented by exercising with BFR. Accordingly, we have provided evidence that BFR augments the exercise-induced molecular signaling response underlying enhancement of K^+ regulation in humans,[91] and cycling for 6 weeks with compared to without BFR caused superior improvements in performance and K^+ regulation during intense exercise in recreationally-active men.[46]

Prior to achieving these successful outcomes, it was carefully considered how to best design the BFR-training protocol. With primary basis in experiments in vitro, a hypothetical model of fluctuations in skeletal muscle oxygen level and ROS production during BFR exercise and subsequent reperfusion rest was drawn (Figure 5). According to this model, the availability of ROS substrate (ie oxygen and NAD(P)H) mainly dictates the amount of ROS that is formed.[86,87] Further, ROS production peaks during the reperfusion phase, where the convective oxygen delivery to tissues is maximal.[86,87,143,145,227] Accordingly, we have later shown that the skeletal muscle blood flow reaches its maximum within the first minute after the end of BFR-exercise, where the tourniquet is deflated (Figure 4). At this stage, it was therefore considered that repeated exercise bouts with

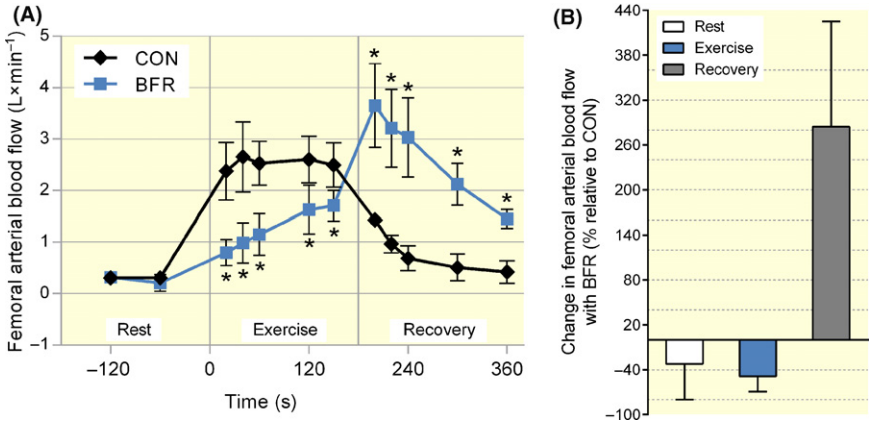

FIGURE 4 Absolute (A) and relative (B) fluctuations in thigh blood flow during and in recovery from moderate-intensity (12 W), single-leg, knee-extensor exercise without (CON) or with blood flow restriction (BFR; ~175 mmHg). Data are expressed as means ± 95% confidence intervals. This figure was reproduced from one of our recent papers.[46]

BFR separated by few minutes with intact blood flow to exercised muscles would be the most effective BFR strategy to augment perturbations in muscle oxygenation, ROS accumulation, and ionic stress. In support, others have found that BFR applied during exercise amplifies metabolic by-product accumulation,[228,229] leading to a more acidic intramuscular environment.[230,231] However, too severe an intracellular acidosis may be detrimental to the post-exercise increase in mRNA levels [168] and adaptability of the ion transport systems to exercise training.[232] Thus, it was considered that exercise bouts with BFR preferably should be several minutes in length to markedly, but only transiently, accumulate H^+ and perturb ion (and redox) homeostasis in exercising muscles.[233] Nevertheless, existence of a threshold of exercise bout duration at which perturbations in redox, ion, and metabolic state may become detrimental to training adaptation remains to be elucidated. Moreover, a moderate exercise intensity may preferably be chosen to avoid premature fatigue caused by BFR. Given these considerations, the BFR-training protocol that we have successfully used in our human experiments consisted of three series of 3 × 2-minute exercise bouts performed at ~60% to 80% of maximal aerobic power, with 1 and 5 minutes of recovery between bouts and series respectively. The tourniquet was inflated 10 s prior to and deflated immediately after each exercise bout. The pressure of the tourniquet was determined in a pilot study, where several exercise sessions were completed with varying degree of BFR (pressure range: 100 to 250 mmHg; n = 2 subjects), whereby the highest tolerable pressure, by which the exercise protocol could be completed, was chosen for our experiments (~175 mmHg).[91]

In summary, exercising with BFR is a potent training concept to promote the molecular mechanisms underlying adaptations in the capacity for K^+ regulation in humans. A key physiological condition likely responsible for the effectiveness of this training model is the alternating nature between a transient, severely-hypoxic and acidic muscular environment interrupted by reperfusion periods with drastically elevated tissue oxygenation. On this basis, it is

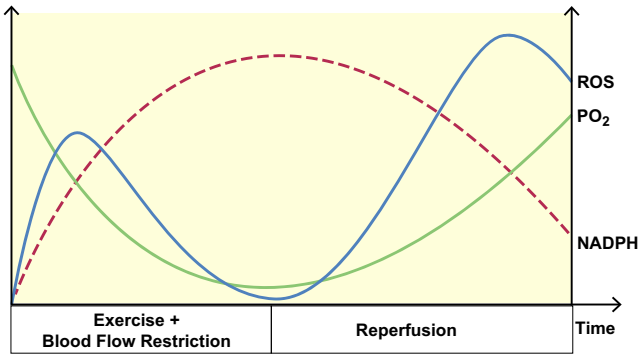

FIGURE 5 Proposed model for fluctuations in reactive oxygen species (ROS) formation, anaerobic glycolytic substrate production for ROS synthesis, and oxygen partial pressure, in skeletal muscle during exercise with blood flow restriction and during the subsequent recovery with intact blood circulation (reperfusion). ROS: blue; PO_2: green; NADPH: hatched red

proposed that designing exercise training interventions, in part, by studying key mechanisms involved in cell adaptation primarily derived from cell culture and animal models is a successful approach to increase the benefits of exercise training on human physical function. In addition, BFR-exercise is an excellent research model to evaluate what molecular signals may drive skeletal muscle adaptation to various stimuli, including physical activity and disease, in humans.

10 | CONCLUSION AND PERSPECTIVES

In humans, improvements in the capacity for K^+ regulation likely result from transient perturbations in both redox, ion, and metabolic homeostasis. Specifically, increases in local oxygen level, ROS production, extracellular [K^+], rate of anaerobic ATP generation, and mRNA expression appear to be key stressors underlying adaptations specific to K^+ regulation, including increases in expression of different Na^+,K^+-ATPase isoforms. In contrast, the level of muscle hypoxia and lactate accumulation per se do not seem essential to these

adaptations in humans. The involvement of alterations in $[Na^+]_i$ and $[Ca^{2+}]_{cyt}$ in mediating these adaptations is presently unclear, although evidence in vitro points to a role of transient oscillations in the concentrations of these ions in regulating Na^+,K^+-ATPase expression. Little is currently understood about how the identified key molecular stressors may be transducing the signal(s) for adaptation in the capacity for K^+ regulation in human skeletal muscle, although a contributory role of AMPK and oxygen-sensing transcription factors has been suggested. Furthermore, enhancement of K^+ regulation by exercise training in humans probably takes place as a result of coordinated increases in expression of catalytic Na^+,K^+-ATPase isoforms and FXYD1 among different fibre types, which appears to be dependent on relative exercise intensity and/or volume, as well as the demand for Na^+/K^+ transport imposed on each fibre type while training. In addition, by the use of blood-flow-restricted exercise as a model to manipulate the molecular stressors underlying training adaptation, it was highlighted how mechanistic insights from animal and cell culture research may be preferably used to make informed decisions about how to design exercise training interventions to maximize improvements in K^+ regulation in humans. The novel information provided in this review paves the way for a better understanding of how to develop interventions, such as exercise training and pharmacological therapies, to improve physical function and potentially hinder the progression of several life-style-related diseases in humans.

ACKNOWLEDGEMENTS

The author thanks Prof David J. Bishop, A/Prof Robyn Murphy, Prof Michael J. McKenna, and Prof Jens Bangsbo for insightful discussions and considerations on this topic.

CONFLICT OF INTERESTS

The author has no conflict of interest that relates to the content of this article.

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How to cite this article: Christiansen D. Molecular stressors underlying exercise training-induced improvements in K^+ regulation during exercise and Na^+,K^+-ATPase adaptation in human skeletal muscle. *Acta Physiol*. 2018;e13196. https://doi.org/10.1111/apha.13196