Regulation of Skeletal Muscle Glucose Uptake: A Focus on Nitric Oxide Synthase and Rac1 Signalling

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ABSTRACT

Muscle contractions and exercise have been shown to potently stimulate muscle glucose uptake via molecular mechanisms that are different to insulin-stimulated glucose uptake. Importantly, although insulin-stimulated glucose uptake is diminished in people with type 2 diabetes, the ability for contractions or exercise to stimulate muscle glucose uptake appears to be preserved. Activating this exercise signalling pathway with novel drugs could theoretically bypass the defective insulin-signalling pathway and lower blood glucose levels in insulin-resistant individuals. However, the exact signalling mechanisms involved require a better understanding.

Several potential regulators of contraction-stimulated glucose uptake have been identified and some degree of redundancy probably exists. Nitric oxide (NO) has been suggested to regulate muscle glucose uptake in rodent and human models. People with type 2 diabetes appear to have a greater reliance on NO to regulate muscle glucose uptake during exercise compared to healthy individuals. Recently, Rac1 has been identified as a novel regulator of muscle glucose uptake during stretch, contraction, and exercise in rodent models. However, the exact mechanisms how both NO and Rac1 regulate glucose uptake are not fully understood. Interestingly, there is some evidence from cell culture studies suggesting an interaction between NO and Rac1 may exist, however, this remains untested in the context of contraction- or exercise-stimulated glucose up in mature muscle models. Therefore, in this thesis, I examined the regulation of skeletal muscle glucose uptake during stretch,

contraction, and exercise with a focus on NOS and Rac1 signalling mechanisms. In particular, the potential for NOS and Rac1 to act via the same signalling pathway was explored.

The pathways activated by stretch have been considered to overlap with contraction signalling. Stretch is known to increase glucose uptake via Rac1, and there is published evidence that stretch increase muscle NO levels. Therefore, in Study 1, an *ex vivo* muscle stretching model was used to examine whether NO also plays a role in stretch-stimulated glucose uptake. Stretch increased glucose uptake in isolated EDL muscles and treatment with the NO synthase (NOS) inhibitors L-NMMA and L-NMMA had no effect on this stretch-stimulated glucose uptake. Likewise, stretch-stimulated glucose uptake was normal in muscles from nNOSμ knockout (KO) and eNOS KO mice. A dissociation between NO and Rac1 was observed given that stretching stimulated an increase in Rac1 signalling but did not increase NOS activity above resting levels. This suggested that activation of Rac1 during stretch does not require NO to regulate glucose uptake.

I was interested to further examine a potential NO and Rac1 interaction in skeletal muscle given there is evidence from previously published studies suggesting that increased levels of NO activate Rac1 in cells in culture. Therefore, in Study 2, I treated isolated muscles with a NO donor (DETA/NO) to increase NO levels, and this increased glucose uptake above resting levels. Addition of a Rac1 inhibitor (Rac1 inhibitor II) completely prevented this DETA/NO stimulation of skeletal muscle glucose uptake. This suggests that NO can

stimulate glucose uptake via a pathway involving Rac1. However, when tested during muscle contraction to increase endogenous NO levels, NOS inhibition did not alter Rac1 signalling during muscle contraction. This suggests that in a more physiological setting, NO does not activate Rac1. Further evidence dissociating a NOS-Rac1 link is suggested by the observation that contraction-stimulated glucose uptake was attenuated by Rac1 inhibition but not by NOS inhibition. The finding that NOS inhibition did not attenuate glucose uptake was surprising as this contrasted with previous studies by our group. Therefore, these results provide further evidence that in regulating muscle glucose uptake, Rac1 signalling does not involve NO. Furthermore, these findings question the hypothesis that NOS plays an important role in the regulation of skeletal muscle glucose uptake during contraction.

Given that findings from Study 1 and Study 2 suggest NOS does not play a role in regulating Rac1 signalling and skeletal muscle glucose uptake, I next turned attention to another promising candidate regulator of Rac1 signalling. RhoGDIα has previously been reported to negatively regulate Rac1 activity in cell culture models. It was important however to examine this in a more physiological model such as whole-body exercise where Rac1 is known to be a major regulator of skeletal muscle glucose uptake. Therefore, in Study 3, RhoGDIα was overexpressed in skeletal muscles of mice to test the hypothesis that exercise-stimulated glucose uptake would be attenuated by the negative action of RhoGDIα on Rac1 signalling. However, increased skeletal muscle RhoGDIα protein levels did not attenuate exercise-stimulated glucose uptake, and Rac1 signalling appeared to be normal. Interestingly, increased levels of Rac1 protein were observed in RhoGDIα overexpressing mice. Therefore,

in contrast to previous cell culture studies where increased levels of RhoGDIα were found to reduce Rac1 signalling, such a negative role for RhoGDIα in a more physiological setting is not so clear. The increase in Rac1 protein levels in RhoGDIα overexpressing mice could have served to maintain Rac1 signalling during exercise and highlights the potential of a compensatory mechanism to protect signalling pathways regulating glucose metabolism.

In summary, findings from this thesis show that during muscle stretching or contraction, NO and Rac1 are not linked. The most striking finding of this thesis is that we were unable to reproduce findings of previous work from our lab since we report that NOS inhibition does not attenuate contraction-stimulated glucose uptake. Given the observed dissociation between NOS and Rac1, another potential regulator of Rac1 signalling, RhoGDIα was examined. In contrast to previous cell culture studies, the role of RhoGDIα as a negative regulator of Rac1 in the context of exercise-stimulated glucose uptake is not clear, since RhoGDIα overexpression induced compensatory changes to other proteins including Rac1 that ultimately did not affect glucose uptake. Further work is required to unravel the complex regulation of Rac1 signalling towards glucose uptake in muscle.

DECLARATION

I, Jarrod Kerris, declare that the PhD thesis entitled “Regulation of skeletal muscle glucose uptake: A focus on nitric oxide synthase and Rac1 Signalling” 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: 09-May-2020

PREFACE

- Chapter Three has been published as the following manuscript: **Kerris JP**, Betik AC, Li J & McConell GK. (2019). Passive stretch regulates skeletal muscle glucose uptake independent of nitric oxide synthase. *Journal of applied physiology* (Bethesda, Md : 1985) 126, 239-245.
- Dr Andrew Betik, Victoria University, performed some of the EDL muscle dissections presented in Chapter Four.
- In Chapter Five, I travelled to the University of Copenhagen, Denmark where the experiments and muscle analyses were performed. Glucose uptake and glycogen assays were performed by Betina Bolmgren and Irene Bech Nielsen. Dr Lykke Sylow and Lisbeth Moller, generated and maintained the RhoGDIα mouse colony, and performed the muscle dissections and treadmill running experiment. I contributed to the treadmill exercise testing and performed all muscle sample processing, protein signalling lab work.

ACKNOWLEDGEMENTS

There have been a number of people who have supported my PhD journey, and without them, completion of this thesis would not have been possible.

Firstly, to my supervisors Prof Glenn McConell and Dr Andrew Betik, for taking me on as a PhD student, challenging my thinking, and encouraging independence throughout the whole process. While there were many experimental setbacks along the way, there were also a lot of positive research experiences that I am very grateful for.

I thank my other fellow McConell lab members during my candidature time. This was a fantastic lab group to be part of. So, a big shout out to Filippe, Scotty, Evelyn, Mary, Chelsea, Raul, Gunveen, and Hong. Thanks also to Steve Lin, often we were sharing lab space and it was great to have your input (and lending me the odd reagent too!).

I was fortunate to undertake some of my lab work in Copenhagen, Denmark. Thanks to Dr Lykke Sylow and Prof Erik Richter for allowing me to visit your lab in Copenhagen and learning from some of the best. Also, to the Sylow lab group, Lisbeth, Steffen, and Estelle, a huge thanks for the help in the lab!

Badger and Hibbert, I'm not sure if sharing a PhD office was a help or hindrance, but the banh mi lunch runs, and Friday nights at the football were always a good move.

Finally, to Sandra. Your support of my efforts to firstly to pursue a PhD, and then writing up over the many years, has probably contributed to this thesis more than I know.

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AICAR	5-aminoimidazole 4-carboxamide ribonucleoside
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
aPKC	Atypical Protein Kinase C
$Ba(OH)_2$	Barium hydroxide
BCA	Bicinchoninic acid
BH_4	(6R)-5,6,7,8-tetrahydrobiopterin
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
CaMKII	Calcium Calmodulin-dependent kinase II
CaMKK	Calcium Calmodulin-dependent kinase kinase
cGMP	Cyclic guanosine monophosphate
cPKC	Conventional Protein Kinase C
DETA/NO	Diethylenetriamine NONOate
DN	Dominant-negative
DTT	Dithiothreitol
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial Nitric Oxide Synthase
FAD	Flavin adenine dinucleotide
GAP	GTPase-activating protein
GDF	GDI-Dissociation factor
GDI	Guanosine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
GTP	Guanosine triphosphate
HAEC	Human aortic endothelial cells
iNOS	Inducible nitric oxide synthase
IRS-1/2	Insulin receptor substrate 1/2
KHB	Krebs-Henseleit buffer
KO	Knock-out
L-NAME	N^G-Nitro-L-arginine methyl ester
L-NMMA	N^G-Monomethyl-L-arginine
MAPK	Mitogen-activated protein kinase
NAC	N-Acetyl Cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
$NaHCO_3$	Sodium bicarbonate
nNOS	Neuronal nitric oxide synthase
nNOSμ	Neuronal nitric oxide synthase mu
nNOSα	Neuronal nitric oxide synthase alpha

nNOSβ	Neuronal nitric oxide synthase beta
nNOSγ	Neuronal nitric oxide synthase gamma
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
PAK	p21 protein (Cdc42/Rac)-activated kinase
PI3-kinase	Phosphoinositide-3 kinase
PIKfyve	FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PVDF	Polyvinylidine difluride
r-AAV	Recombinant adeno-associated virus
Rac1	RAS-related C3 botulinum substrate 1
RhoA	Ras homolog family member A
RhoGDI	RHO protein GDP dissociation inhibitor
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sGC	Soluble guanylyl cyclase
siRNA	Small interfering RNA
SNP	Sodium nitroprusside
SR	Sarcoplasmic reticulum
T2D	Type 2 Diabetes

TBC1D1	tre-2/USP6, BUB2, cdc16 domain family member 1
TBC1D4	tre-2/USP6, BUB2, cdc16 domain family member 4
TBST	Tris-buffered saline
$VO_{2\ peak}$	Peak oxygen consumption

CHAPTER ONE: INTRODUCTION

1.1. Type 2 Diabetes: A Global Health Problem

The prevalence of diabetes poses a significant challenge to health and healthcare systems around the world. Diabetes is a chronic condition which can cause serious damage to many bodily systems and the long-term health consequences of the disease can include cardiovascular disease, renal failure, neurodegeneration, limb amputation, and blindness. A recent report by the International Diabetes Federation estimated that between the years 2015 to 2040 the number of adults living with diabetes globally would dramatically increase by more than 50% (Ogurtsova *et al.*, 2017). Diabetes carries a significant economic burden, with increased healthcare expenditure, government subsidises, and loss in workplace productivity estimated to cost up to $14 billion in Australia alone (Australian Government, 2015).

Type 2 diabetes is the predominant type of diabetes, accounting for ~90% of diabetes cases and is characterised by elevated fasting blood glucose levels due to deficiencies in both insulin production by the pancreas and the ability of insulin to stimulate glucose uptake in peripheral tissues (Chatterjee *et al.*, 2017). To normalise blood glucose levels, people with type 2 diabetes largely rely on hypoglycaemic drugs or insulin therapies, however, the long-term efficacy of current drug therapies is limited and not without the risk of undesired side-effects (DeFronzo *et al.*, 2013). Therefore, the development of novel therapeutics that lower blood glucose levels are needed.

1.2. Exercise as a model for drug discovery

Skeletal muscle is an important site for the regulation of blood glucose, accounting for up to 80% of insulin-stimulated glucose disposal (DeFronzo *et al.*, 1981). Like insulin, exercise is also a potent stimuli for skeletal muscle glucose uptake, however, importantly, the ability of exercise to stimulate muscle glucose uptake in people with Type 2 Diabetes is mostly normal (Martin *et al.*, 1995; Kingwell *et al.*, 2002). Given that people with type 2 diabetes have impaired insulin-stimulated muscle glucose uptake, these studies suggest that despite defects in the insulin signalling pathway the "glucose transport machinery" remains intact in these individuals and the exercise-mediated signalling pathway is maintained. Despite this knowledge, the long-term adherence to regular exercise programs is often limited, either due to motivational factors or the associated morbidity with worsening diabetic complications (Praet & van Loon, 2009). Theoretically, intracellular signalling proteins that are activated by exercise could be pharmacologically activated, and in turn, mimic the blood-glucose lowering effect of exercise in diabetic individuals.

Despite significant research efforts over the last several decades, the exact mechanisms by which exercise regulates skeletal muscle glucose uptake is not fully understood. Nonetheless, a number of potential regulators have been identified including calcium, reactive oxygen species (ROS), AMPK, p38 MAPK, TBC1D1/4, nitric oxide synthase (NOS) and Rac1 (Richter & Hargreaves, 2013). While the literature surrounding these signalling intermediates will be discussed within this thesis, the primary focus will be

to explore NOS and Rac1 signalling. There is causal evidence in human studies showing that NOS is required for the regulation of muscle glucose uptake during exercise (Bradley *et al.*, 1999; Kingwell *et al.*, 2002). This was demonstrated by using pharmacological NOS inhibitors which also attenuated the increase in muscle glucose uptake during exercise. Importantly, there is some evidence that NOS regulates muscle glucose uptake during exercise in people with type 2 diabetes to a greater extent than healthy people (Kingwell *et al.*, 2002), highlighting the therapeutic potential for targeting the NOS signalling pathway. Interestingly, Rac1, a novel muscle glucose uptake regulator (Sylow *et al.*, 2016) is potentially linked to NOS signalling. Indeed, there is evidence in muscle cell culture experiments showing that increasing NO levels can increase Rac1 activity (Godfrey & Schwarte, 2010). Whether NOS activates Rac1 in the context of skeletal muscle glucose uptake has not yet been investigated and will be explored in this thesis.

1.3. Overview of Nitric Oxide (NO) and Rac1

1.3.1. NO and NO Synthase (NOS)

Nitric oxide (NO) is a short-lived gaseous molecule that participates in numerous signalling pathways and cellular functions (Stamler & Meissner, 2001). In mammalian cells, NO is predominantly produced by NO synthase (NOS) enzymes of which three isoforms have been identified: Neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial (eNOS). Despite this nomenclature, NOS are not unique to a specific cell type and encompass

a wide tissue distribution. In addition, alternative mRNA splicing of the nNOS gene results in the translation of nNOSα, nNOSβ, nNOSγ, and nNOSμ splice variants, where nNOSα is the full-length isozyme (Brenman *et al.*, 1997). nNOS and eNOS are considered to be constitutively expressed in an inactive form, and require the Ca^{2+}-dependent binding of calmodulin to activate NO synthesis, however, there is evidence of expressional regulation of these isoforms (Forstermann *et al.*, 1998). iNOS expression is predominantly induced by cytokines or bacterial products and can produce high levels of NO in a Ca^{2+}-independent manner (MacMicking *et al.*, 1997). Additionally, the existence of a mitochondrial NOS isoform has been suggested by some studies, however, this remains controversial (Lacza *et al.*, 2009). In skeletal muscle, nNOSμ is suggested to be the main source of NO during contractions (Lau *et al.*, 2000).

Additionally, NO can also be generated by pathways independent of NOS. Nitrate (NO_3^-) and nitrite (NO_2^-) anions were originally considered as inert end-products of NO oxidation, however, it is now accepted that these anions can be converted back to NO (Lundberg *et al.*, 2008). NO_3^-, found in food, can be converted to NO_2^- by bacteria in the mouth, then under conditions of reduced oxygen tension or acidosis, NO_2^- can be reduced to NO via several enzymatic and non-enzymatic mechanisms thereby ensuring NO production is maintained where NOS-derived NO production may be impaired (Lundberg *et al.*, 2008). However, NO-mediated glucose uptake during contraction and exercise appears to be NOS-dependent (Hong *et al.*, 2014).

1.3.2. Rac1

The Rho family small GTPase Rac1 (RAS-related C3 botulinum substrate 1) is considered to play a central role in the regulation of actin cytoskeleton dynamics (Ridley, 2006). Rac1 functions as a molecular switch, alternating between an active GTP (guanosine-5'-triphosphate)-bound form, and an inactive GDP (guanosine-5'-diphosphate)-bound form. Guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP and thus turn the system "on", whereas the slow intrinsic GTPase activity is enhanced by GTPase-activating proteins (GAPs) which promote GTP hydrolysis and turn the system "off". In addition, another level of regulation is afforded by guanine dissociation inhibitors (GDIs) that maintain the GTPase in a soluble inactive pool. RhoGDIα is expressed in skeletal muscle (Zalcman *et al.*, 1996; Sakuma *et al.*, 2008) and is the GDI that targets Rac1 (DerMardirossian *et al.*, 2004). Dissociation of the GTPases from complex with GDI's is facilitated by GDI-dissociation factors (GDF). (Figure 1.1). However, understanding of these factors, particularly with respect to muscle glucose uptake is limited.

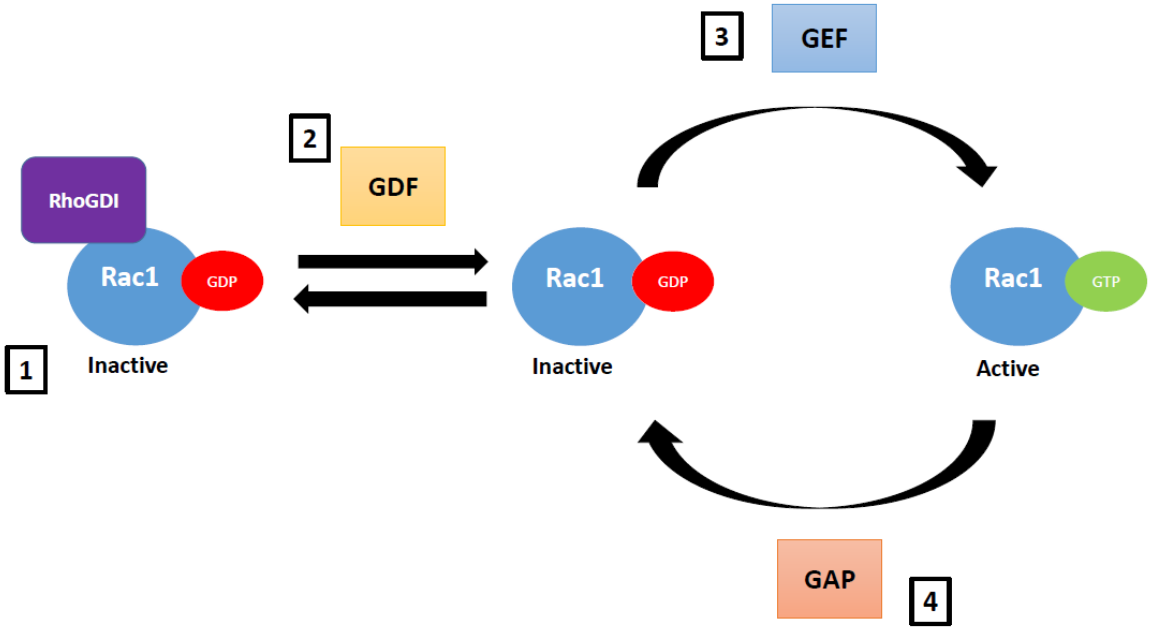

Figure 1.1 Overview of the modulation of Rac1 activity. (1) GDP-bound Rac1 is sequestered in an inactive complex by Rho guanine dissociation inhibitor (RhoGDI). (2) GDI dissociation factors (GDF) stimulate the dissociation of Rac1 from complexation with RhoGDI. (3) Guanine nucleotide exchange factors (GEF) catalyse the release of bound GDP allowing for the binding of GTP and subsequent activation and interaction with effector proteins. The slow intrinsic GTPase activity is potentiated by GTPase-activating proteins (GAP) thus returning the active Rac1 to an inactive GDP-bound form and terminating its signal (4).

1.4. Definition of exercise models

Within the scientific literature, studies investigating the mechanistic signalling of exercise-stimulated glucose uptake cover a range of experimental models and before proceeding with this thesis, it is important to define the terms describing the key models utilised. “Exercise” will refer to the *in vivo* setting whereby rodents or humans undertake whole-body exercise (e.g., treadmill running or cycling). “Contraction” will refer to either rodent skeletal muscles or cells in culture undergoing contractions, usually induced by electrical stimulations either *in vitro*, *ex vivo*, or *in situ* settings. “Stretch” will refer to the application of passive tension (stretching) to resting muscles and for the purpose of this thesis includes *in vitro* and *ex vivo* settings of cells in culture or rodent skeletal muscle.

1.5. Thesis Overview

This thesis will explore the role of two promising signalling proteins NOS and Rac1, in the context of muscle glucose uptake during muscle stretching, contractions, and exercise by using rodent models. A review of the literature (Chapter Two) will briefly highlight the potential signalling candidates known to date, with a more in-depth review of the NOS and Rac1 literature. In experimental Chapters Three and Four, the potential link between NOS and Rac1 signalling towards glucose uptake will be investigated, and in Chapter Five, the upstream regulation of Rac1 by RhoGDIα during exercise will be explored.

CHAPTER TWO: LITERATURE REVIEW

2.1. Muscle glucose uptake during exercise

The rate of skeletal muscle glucose uptake is markedly increased during exercise. This is due to coordinated increases in i) capillary perfusion and glucose delivery to the muscle cell, ii) glucose uptake via the movement of intracellular glucose transport proteins to the plasma membrane and t-tubules, and iii) phosphorylation of glucose to glucose-6-phosphate within the cell, where the glucose becomes trapped within the cell for further metabolism. It is generally considered that these steps operate collectively and their relative contribution is dependent on the conditions of the contracting muscle (Wasserman & Halseth, 1998). This thesis will focus on the intracellular signalling towards glucose transport during exercise and muscle contraction.

The transport of glucose into muscle cells is facilitated by the actions of the glucose transport proteins GLUT1 and GLUT4. GLUT1 is constitutively maintained in low levels of skeletal muscle membranes and is primarily involved in the regulation of basal glucose uptake (Mueckler, 1994). GLUT4 is the primary GLUT involved in exercise-stimulated glucose uptake. Indeed, contraction- and exercise-stimulated skeletal muscle glucose uptake is markedly reduced in GLUT4-null mice (Ryder *et al.*, 1999; Zisman *et al.*, 2000; Fueger *et al.*, 2007; Howlett *et al.*, 2013), whereas muscle contraction-stimulated glucose uptake is increased in mice overexpressing GLUT4 (Hansen *et al.*, 1995). GLUT4 is mostly retained

in intracellular compartments under basal conditions (Bryant *et al.*, 2002), and exercise causes GLUT4 to translocate to the cell surface. Indeed, GLUT4 content in membrane and t-tubule fractions of muscle homogenates is markedly increased following contractions in rodents (Roy & Marette, 1996; Ploug *et al.*, 1998; Ito *et al.*, 2006), and exercise in humans (Kristiansen *et al.*, 1996, 1997; Kennedy *et al.*, 1999; Thorell *et al.*, 1999). Furthermore, sarcolemma GLUT4 content demonstrates a high correlation with glucose uptake during contractions in rodents (Derave *et al.*, 1999) and exercise in humans (Kristiansen *et al.*, 1997).

2.2. Insulin versus Contraction Signalling

Although insulin and contractions both stimulate GLUT4 translocation and glucose uptake, the signalling pathways arising from these stimuli are known to be at least proximally distinct, and possibly signalling towards different intracellular GLUT4 pools (Douen *et al.*, 1990; Coderre *et al.*, 1995). A dissociation between insulin and contraction signalling was initially proposed based on the observations that contractions can increase glucose uptake without the presence of insulin, and, the effects of maximal insulin and contraction stimulation on glucose uptake are additive (Ploug *et al.*, 1984; Nesher *et al.*, 1985; Wallberg-Henriksson *et al.*, 1988). At the proximal level, insulin signalling is initiated by the interaction of insulin with its cell surface receptor leading to the activation of IRS-1/2 and PI3-kinase. However, exercise does not activate these signalling proteins (Goodyear *et al.*, 1995; Brozinick & Birnbaum, 1998). In accord, inhibition of the insulin-signalling cascade

at the level of PI3-kinase has been shown to impair insulin-stimulated glucose uptake whereas contraction-stimulated glucose uptake is maintained (Lee *et al.*, 1995; Lund *et al.*, 1995). These findings were further confirmed in a study using muscle-specific insulin receptor knockout mice. The mice were found to exhibit normal contraction-stimulated glucose uptake while insulin-stimulated glucose uptake was nearly abolished (Wojtaszewski *et al.*, 1999a). Importantly, while insulin-dependent glucose uptake is diminished in people with type 2 diabetes and insulin-resistant rodent models, the ability of contractions or exercise to stimulate GLUT4 translocation and glucose uptake appears to be normal (Brozinick *et al.*, 1994; Kennedy *et al.*, 1999; Kingwell *et al.*, 2002). Preservation of the exercise signalling pathway in insulin-resistant muscle, therefore, has exciting drug-target potential to lower blood glucose levels in people with type 2 diabetes.

2.3. Molecular regulators of glucose uptake during exercise and contraction

While the molecular mechanisms regulating GLUT4 translocation and glucose uptake in response to insulin are largely defined, the contraction-dependent mechanisms are not clear. Dissecting the exact signalling pathways(s) involved has proven challenging as a high degree of redundancy is likely to exist whereby the loss of one signalling protein is compensated by others. Furthermore, no single molecule is responsible for the entirety of signalling towards GLUT4 translocation and glucose uptake, and the relative contribution of regulators involved likely depends on factors such as the metabolic state of the muscle and

exercise/contraction intensity and duration. Several signalling mechanisms have been proposed to regulate skeletal muscle glucose uptake during exercise including mechanical stress, calcium, nitric oxide (NO), reactive oxygen species (ROS), AMPK, p38 MAPK, TBC1D1/4, and Rac1 (Richter & Hargreaves, 2013) as shown in Figure 2.1.

For many years, concepts of the signalling pathways involved proposed two levels of regulation. One being feed-forward signals related to the increase in Ca^{2+} concentrations with membrane depolarisation and the other level being feed-back signals relating to the metabolic status of the muscle (Richter & Hargreaves, 2013). More recent evidence, however, suggests that signalling towards glucose uptake during exercise can be divided into signals arising from mechanical-dependent or metabolic-dependent mechanisms (Jensen *et al.*, 2014; Sylow *et al.*, 2015; Sylow *et al.*, 2017b). These signalling factors will be briefly discussed within this literature review with a primary focus on the NO and Rac1 signalling pathways and the potential interaction between these signalling candidates.

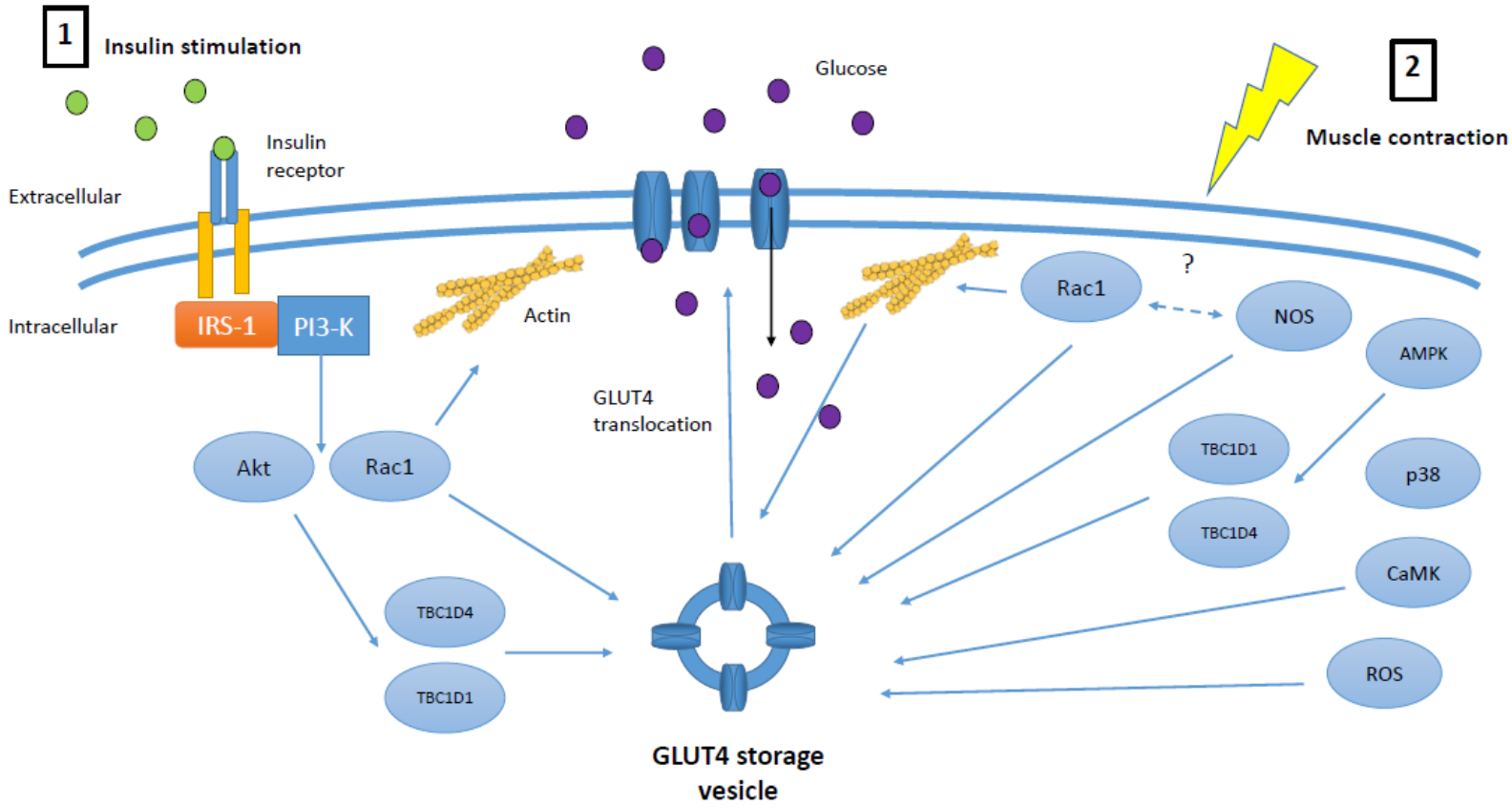

Figure 2.1 Overview of muscle glucose transport in response to insulin and contraction stimulation (continued on the following page)

Figure 2.1 Overview of muscle glucose transport in response to insulin and contraction stimulation. Insulin (1) and muscle contractions (2) stimulate intracellular signalling factors that promote the translocation of GLUT4 to the cell membrane, facilitating the entry of glucose into the cell. These stimuli operate via signalling pathways that are proximally distinct. Convergence points possibly exist at the levels Rac1, and TBC1D1/4. IRS-1, insulin receptor substrate 1. PI3-K, Phosphoinositide kinase 3. TBC1D1, TBC1 domain family member 1. TBC1D4, TBC1 domain family member 4. Rac1, p21-Rac1. NOS, nitric oxide synthase. AMPK, AMP-activated protein kinase. CaMK, Ca^{2+}/calmodulin-dependent protein kinase. ROS, reactive oxygen species.

2.3.1. Mechanical stress

During exercise, mechanical stresses are imposed on the contracting muscles, and there is evidence that mechanical stress in the form of passive stretching stimulates glucose uptake in muscles *ex vivo* (Ihlemann *et al.*, 1999b; Chambers *et al.*, 2009; Jensen *et al.*, 2014; Sylow *et al.*, 2015), *in situ* (Ito *et al.*, 2006), and in cells *in vitro* (Iwata *et al.*, 2007; Iwata *et al.*, 2009). Studies have shown that glucose uptake is attenuated (~20-40%) when force development (i.e., mechanical stress) is reduced during electrical stimulations. This has been demonstrated in muscles treated with myosin ATPase inhibitors (to prevent cross-bridge cycling) or by shortening the muscle resting length so that cross-bridge interactions are minimised (Ihlemann *et al.*, 1999b; Jensen *et al.*, 2014; Sylow *et al.*, 2015).

An emerging hypothesis suggests that muscle contractions and exercise activate parallel signalling arms, one that is linked to mechanical stress and the other linked to metabolic demand (Jensen *et al.*, 2014) (Figure 2.2). Studies have employed stretching models to delineate the signalling proteins potentially involved. Indeed, while stretching stimulates glucose uptake, activation of the metabolic signalling intermediate AMPK is not caused by stretch (Chambers *et al.*, 2009; Jensen *et al.*, 2014). Published studies to date have suggested that ROS, Rac1, and p38 MAPK are involved in this mechanically-linked signalling component of glucose uptake (Chambers *et al.*, 2009; Sylow *et al.*, 2015). Additionally, there is evidence that muscle stretching activates NOS in mature muscle (Tidball *et al.*, 1998), and this should be further investigated in the context of glucose uptake.

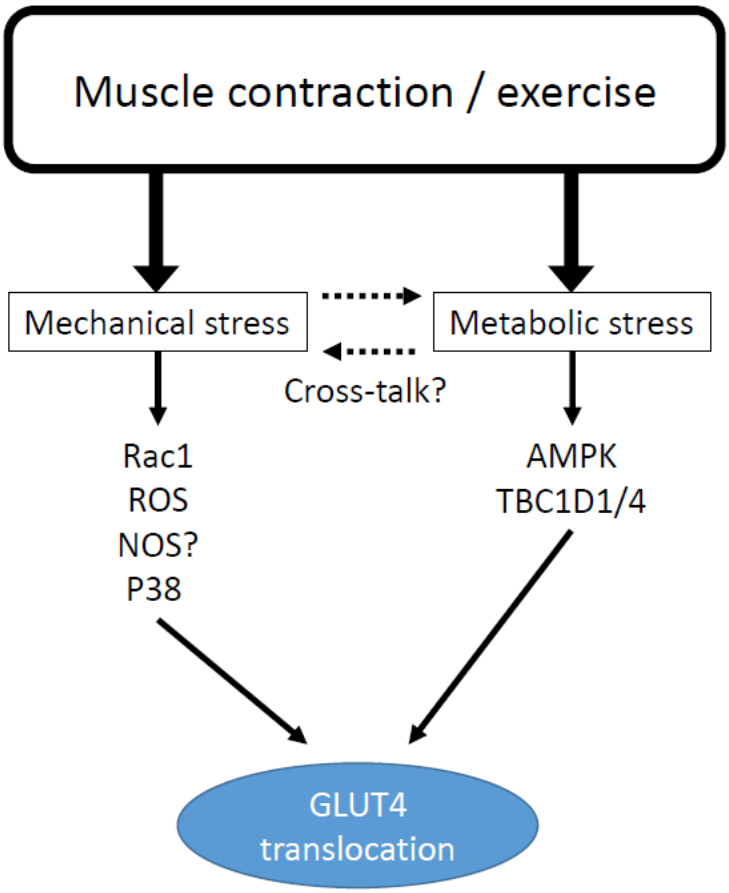

Figure 2.2 Overview of mechanical- and metabolic-stress pathways proposed to be activated by contraction and exercise.

One study, however, reported that electrically-stimulated glucose uptake was normal despite the complete prevention of force development by using ATPase inhibitors (Sandstrom *et al.*, 2007). The lack of effect on glucose uptake in this study could be due to

the higher stimulation intensity used compared to previous studies (Ihlemann *et al.*, 1999b; Jensen *et al.*, 2014; Sylow *et al.*, 2015). It is likely that the contribution of mechanical-signalling pathways to glucose uptake during contractions is dependent on the intensity of the contractions and other signalling factors in response to metabolic stress are at play. This notion is supported in a series of experiments where isolated muscles were electrically stimulated to contract at different intensities while in the presence of myosin ATPase inhibitors to prevent force production during contractions (Jensen *et al.*, 2014). When force production was prevented by myosin ATPase inhibition during electrical stimulations, glucose uptake was fully blocked during low-intensity stimulation, partially blocked at moderate intensity, and was normal at higher intensities (Jensen *et al.*, 2014). This suggests that the at lower contraction/exercise intensities, mechanical signalling proteins may serve a greater contribution to the glucose uptake signalling pathway than at higher contraction/exercising intensities where these same proteins may not be required.

2.3.2. Calcium, PKC, and CaMK

The increase in cytoplasmic calcium (Ca^{2+}) concentration with muscle contraction has long been postulated as a critical regulator of contraction-mediated glucose uptake by way of a feed-forward mechanism. Early studies demonstrated that caffeine-stimulated sarcoplasmic reticulum (SR) Ca^{2+} release increased glucose uptake in amphibian and mammalian skeletal muscle (Holloszy & Narahara, 1967; Youn *et al.*, 1991; Wright *et al.*, 2004). Consistent with this, prevention of calcium release from the SR with Dantrolene was

shown to inhibit caffeine-induced glucose uptake (Youn *et al.*, 1991). Since elevations in cytoplasmic calcium concentrations with each muscle contraction are transient, but glucose transport persists for a prolonged period following the cessation of contractile activity, rather than directly mediating glucose uptake it is likely that Ca^{2+} achieves this by activating downstream intracellular factors.

Whether Ca^{2+}-dependent glucose transport involves other signalling pathways such as AMPK is not clear. Earlier studies reported that stimulation of Ca^{2+} release by caffeine did not induce changes in nucleotide turnover, or AMPK activation (Youn *et al.*, 1991; Wright *et al.*, 2004). However, more recent studies suggest an association between Ca^{2+} release and AMPK activation (Jensen *et al.*, 2007a; Raney & Turcotte, 2008; Egawa *et al.*, 2011), likely due to the increased energy demand required by Ca^{2+}-ATPase sarcoplasmic reticulum-dependent reuptake of Ca^{2+} (Smith *et al.*, 2013; Jensen *et al.*, 2014), rather than the direct effect of Ca^{2+} per se.

Early studies demonstrated that muscle contractions or exercise in rodents promoted the redistribution and phosphorylation of Ca^{2+}-dependent conventional PKC (cPKC) from cytosolic to membrane fractions of skeletal muscle (Richter *et al.*, 1987; Cleland *et al.*, 1989). Although downregulation or inhibition of cPKC isoforms has been shown to attenuate contraction-stimulated glucose (Henriksen *et al.*, 1989; Cleland *et al.*, 1990; Wojtaszewski *et al.*, 1998; Ihlemann *et al.*, 1999a), contraction-stimulated glucose uptake was shown to be normal despite deletion of the predominant cPKC isoform, cPKCα (Jensen *et al.*, 2009).

Ca^{2+}-independent, atypical PKC (aPKC) isoforms have been implicated in insulin-stimulated glucose uptake in skeletal muscle (Farese *et al.*, 2007) and are also activated by exercise in humans (Beeson *et al.*, 2003; Perrini *et al.*, 2004; Richter *et al.*, 2004; Rose *et al.*, 2004), however, rodent studies have questioned whether aPKC's play a role in contraction/exercise-stimulated glucose uptake. Indeed, although skeletal muscle aPKC activity in rodents increases following treadmill exercise (Chen *et al.*, 2002; Sajan *et al.*, 2010), it has been shown that loss of aPKC did not impair skeletal muscle glucose uptake during exercise (Sajan *et al.*, 2010), or contractions (Yu *et al.*, 2015). These studies suggest that it is unlikely that PKC's are involved in the regulation of glucose transport during exercise.

Exercise is known to increase phosphorylation of calcium/calmodulin-dependent kinase II (CaMKII) in skeletal muscle of humans (Rose & Hargreaves, 2003; Rose *et al.*, 2006; Combes *et al.*, 2015) and rodents (Wright *et al.*, 2004). Inhibition of CaMKII or the upstream CaMK kinase (CaMKK) was reported to attenuate caffeine- and contraction-stimulated glucose uptake in rodent muscles (Wright *et al.*, 2004; Wright *et al.*, 2005; Jensen *et al.*, 2007b; Witczak *et al.*, 2007). In support, electroporation of an inhibitory CaMKII into tibialis anterior muscle of mice resulted in an attenuation of contraction-stimulated glucose uptake (Witczak *et al.*, 2010). Therefore, activation of CaMKII may function to mediate muscle glucose uptake during exercise and the mechanisms involved requires further work.

2.3.3. AMP-activated protein kinase (AMPK)

AMPK has long been considered an important regulator of cellular energy homeostasis and has received considerable research attention over the years (Kjobsted *et al.*, 2018). Increased AMPK activity with contractions or exercise has been well described in rodent (Winder & Hardie, 1996; Vavvas *et al.*, 1997; Jorgensen *et al.*, 2004; Merry *et al.*, 2010c) and human studies (Chen *et al.*, 2000; Fujii *et al.*, 2000; Wojtaszewski *et al.*, 2000b; Stephens *et al.*, 2002; Chen *et al.*, 2003). The AMPK-activating compound 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) stimulates glucose uptake in resting skeletal muscle, while this effect is ablated in AMPK-knockout and AMPK-dominant negative mouse models (Jorgensen *et al.*, 2004; Maarbjerg *et al.*, 2009; Steinberg *et al.*, 2010). Although these studies suggest a link between AMPK activation and glucose uptake, the involvement of AMPK in regulating glucose uptake during contraction or exercise is controversial.

Human studies published to date have failed to establish an association between AMPK and glucose uptake during exercise. Indeed in one study, AMPK activity was not increased during the first 2 hours of prolonged low-intensity cycling exercise (~45% $VO_{2\ peak}$) despite a greater than 10-fold increase in leg glucose uptake (Wojtaszewski *et al.*, 2002). In another study, exercise failed to increase AMPK activity when performed following a 10-day exercise training intervention while only a slight reduction in exercise-stimulated glucose uptake was observed (McConell *et al.*, 2005). Similar results were reported following a 12-

week exercise training intervention where there was no increase in total AMPK activity despite increased muscle glucose uptake during exercise (Mortensen *et al.*, 2013). It is important to note, however, that these studies report on the association between AMPK activity and exercise-stimulated glucose and does not allow for interpretation of a cause and effect of AMPK activity on glucose uptake. Nonetheless, the weight of these important human data is not diminished and the AMPK hypothesis has been understandably questioned in a recently published perspective (McConell, 2020).

Rodent studies involving genetically modified AMPK mice to examine the function of AMPK signalling mechanisms have been conflicting. Indeed, in AMPK knockout or dominant-negative mouse models, exercise- or contraction-mediated glucose uptake has been reported to be reduced in some studies (Mu *et al.*, 2001; Fujii *et al.*, 2005; Jensen *et al.*, 2008; Lefort *et al.*, 2008; Lee-Young *et al.*, 2009; Abbott *et al.*, 2011; O'Neill *et al.*, 2011; Sylow *et al.*, 2017b), but not in others (Barnes *et al.*, 2004; Jorgensen *et al.*, 2004; Fujii *et al.*, 2007; Witczak *et al.*, 2007; Maarbjerg *et al.*, 2009; Merry *et al.*, 2010c; Steinberg *et al.*, 2010; Lantier *et al.*, 2014; Fentz *et al.*, 2015; Sylow *et al.*, 2017b; Kjobsted *et al.*, 2019). Recently, it has been highlighted that some studies reporting that contraction-stimulated glucose uptake is attenuated in AMPK knockout mice have tended to measure muscle glucose uptake in the period following contraction, and not during (Kjobsted *et al.*, 2019). Nonetheless, there is building evidence that whilst AMPK may not be important during contraction, the increase in insulin-stimulated glucose uptake in the period following contraction likely involves AMPK (Kjobsted *et al.*, 2015; Kjobsted *et al.*, 2017; Oki *et al.*, 2018; Wang *et al.*, 2018;

Kjobsted *et al.*, 2019). However, this mechanism requires further investigation in human studies.

Another approach to probe the potential role of AMPK has been to examine signalling mechanisms both up- and down-stream of AMPK. Contraction- and exercise-induced AMPKα2 activity is blunted by the deletion of the predominant AMPK kinase LKB1 (Koh *et al.*, 2010; Jeppesen *et al.*, 2013), and contraction-stimulated glucose uptake is attenuated in LKB1 KO mice (Sakamoto *et al.*, 2005; Koh *et al.*, 2010). However, LKB1 knockout mice exhibit normal muscle glucose uptake during exercise (Jeppesen *et al.*, 2013). It should be noted that in addition to targeting AMPK several other kinases are also targeted by LKB1 (Lizcano *et al.*, 2004). This includes sucrose nonfermenting AMPK-related kinase (SNARK), a protein which has been suggested by one study to regulate contraction-stimulated glucose uptake (Koh *et al.*, 2010). In another study which looked at AMPK downstream targets, pharmacological inhibition of the AMPK substrate FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) was shown to attenuate contraction-stimulation glucose uptake (Liu *et al.*, 2013). However, investigations using PIKfyve knockout models should be carried out, as well as *in vivo* exercise models.

2.3.4. Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are low molecular weight free radicals derived from molecular oxygen. ROS are produced at low levels in resting skeletal muscle and increased with contractile activity and stretching (Pattwell *et al.*, 2004; Sandstrom *et al.*, 2006;

Chambers *et al.*, 2009; Merry *et al.*, 2010c; Henriquez-Olguin *et al.*, 2019). While classically considered deleterious and associated with a number of pathologies, ROS are now recognised as essential mediators of cell signalling under normal physiological conditions.

Exogenous ROS have been shown to increase basal glucose uptake in isolated rodent muscle (Toyoda *et al.*, 2004; Higaki *et al.*, 2008; Jensen *et al.*, 2008), and this effect was attenuated by the concomitant exposure of ROS scavengers (Toyoda *et al.*, 2004; Higaki *et al.*, 2008). The antioxidant N-acetylcysteine (NAC) has been shown to attenuate ROS production and glucose uptake during *ex vivo* contractions (Sandstrom *et al.*, 2006; Merry *et al.*, 2010b; Merry *et al.*, 2010c) as well as reductions in basal glucose uptake (Merry *et al.*, 2010b). Furthermore, several ROS-scavenging agents were shown to completely block the increase in glucose uptake of mouse EDL muscle in response to passive stretching (Chambers *et al.*, 2009). In support, mouse models lacking functional NADPH oxidase 2 were found to also lack exercise-stimulated muscle ROS production and exhibit significantly attenuated muscle glucose uptake during exercise (Henriquez-Olguin *et al.*, 2019). While these studies suggest that ROS are essential for the increase in glucose uptake during contractions or exercise, NAC administration failed to attenuate skeletal muscle glucose uptake in perfused rat hindlimb during contractions *in situ* (Merry *et al.*, 2010a). Furthermore, systemic infusion of NAC during moderate-intensity cycling exercise in humans (~60% $VO_{2\ peak}$) did not affect whole-body glucose disposal (Merry *et al.*, 2010d). In these studies, however, there was less of a shift in oxidant status and ROS signalling compared to other experimental models used

which could explain the lack of NAC effect, and highlight that ROS signalling towards glucose uptake is likely dependent on higher exercising intensities.

2.3.5. Mitogen activated protein kinases (MAPK)

Several proteins in the MAPK family, including ERK 1/2, SEK1, JNK1, and p38 are activated during muscle contractile activity (Aronson *et al.*, 1997; Ryder *et al.*, 2000; Richter *et al.*, 2004). However, inhibition of the upstream ERK kinase (MAP/ERK kinase) with PD-98059 has been shown to prevent ERK phosphorylation without affecting glucose uptake during contractions in rats (Hayashi *et al.*, 1999; Wojtaszewski *et al.*, 1999b). In addition, deletion of JNK1 did not affect contraction-stimulated glucose uptake in EDL and soleus muscles (Witczak *et al.*, 2006). Thus, it is unlikely that ERK and JNK are involved in regulating glucose uptake during contractions.

While some studies have suggested that p38 MAPK is involved in regulating muscle glucose uptake during contraction, there are conflicting reports. Pharmacological inhibition of p38 has been shown to attenuate AICAR- and contraction-stimulated glucose uptake (Somwar *et al.*, 2000; Lemieux *et al.*, 2003), however, the inhibitor used in those studies (SB203580) has been reported to have p38 MAPK-independent effects (Eyers *et al.*, 1999; Antonescu *et al.*, 2005; Ribe *et al.*, 2005; Turban *et al.*, 2005). Using a more selective p38 MAPK inhibitor (A304000), stretch-stimulated glucose uptake was reported to be completely blocked in the presence of the inhibitor (Chambers *et al.*, 2009). However, in another study, the observation of normal stretch-stimulated glucose uptake despite the complete blockade

of p38 MAPK signalling by another inhibitor (VX-702) (Jensen *et al.*, 2014) indicates a clear dissociation between glucose uptake and p38 activation. In an earlier study, contraction-stimulated glucose uptake was normal in muscles where the predominant p38 MAPK isoform (p38γ) was overexpressed (Ho *et al.*, 2004). Nonetheless, p38 MAPK-null mice as well as using selective p38 inhibitors during contraction would be valuable in clarifying the role of p38 MAPK in regulating muscle glucose uptake. Overall, a role for p38 MAPK in regulating glucose uptake during exercise is yet to be definitively established.

2.3.6. TBC1D1 and TBC1D4

Tre-2/BUB2/cdc 1 domain family 1 (TBC1D1) and its paralogue TBC1D4 (also known as Akt substrate of 160 kDa, AS160) are Rab-GTPase activating proteins (Rab GAPs) (Sakamoto & Holman, 2008) implicated in glucose uptake. TBC1D1/4 proteins negatively regulate specific GLUT4-associated Rab proteins resulting in the retention of GLUT4 to intracellular compartments. Phosphorylation of TBC1D1/4 at specific sites relieves the GAP activity towards Rab allowing for activation of Rab and an increase in GLUT4 translocation and glucose uptake (Sano *et al.*, 2003; Eguez *et al.*, 2005; Sano *et al.*, 2007; Chavez *et al.*, 2008).

Several studies have demonstrated that contractions and exercise increase TBC1D1 and TBC1D4 phosphorylation on Akt- and AMPK-phosphorylation sites in rodent and human skeletal muscle (Deshmukh *et al.*, 2006; Kramer *et al.*, 2006; Funai & Cartee, 2009; Frosig *et al.*, 2010; Treebak *et al.*, 2010; Jessen *et al.*, 2011; Treebak *et al.*, 2014).

Electroporation of non-phosphorylatable mutants of TBC1D1 or TBC1D4 (Kramer *et al.*, 2006; An *et al.*, 2010; Vichaiwong *et al.*, 2010), or a TBC1D1 mutant unable to bind calmodulin (Kramer *et al.*, 2007) in mouse tibialis anterior muscle, has been shown to attenuate contraction-stimulate glucose uptake. Likewise, mice lacking TBC1D1 have been shown to have attenuated contraction- and exercise-stimulated glucose uptake (Szekeres *et al.*, 2012; Stockli *et al.*, 2015). However, reduction in glucose uptake in the TBC1D1 knockout mice may be partly explained by a reduced muscle GLUT4 content (~40-50%).

2.3.7. Rac1

Early cell culture experiments first reported a role for Rac1 in regulating glucose uptake via the insulin-dependent signalling pathway. Transfection of a dominant-negative Rac1 in L6 myotubes was shown to block GLUT4 translocation in response to insulin (Khayat *et al.*, 2000), whereas overexpression of constitutively active Rac1 was reported to induce GLUT4 translocation and glucose uptake in muscle cells (Ueda *et al.*, 2010; Chiu *et al.*, 2013). In a series of more recent studies, Sylow and colleagues have demonstrated that Rac1 plays a role in contraction- and exercise-mediated glucose uptake in rodent models. Skeletal muscle Rac1 activity is increased in rodents during contractions and stretch *ex vivo* (Sylow *et al.*, 2013b; Sylow *et al.*, 2015), during exercise *in vivo* (Sylow *et al.*, 2013b), and in humans during exercise (Sylow *et al.*, 2013b). Inhibition of Rac1 by pharmacological inhibitors has been shown to attenuate contraction-stimulated glucose uptake in isolated EDL and soleus muscle (Sylow *et al.*, 2013b; Sylow *et al.*, 2017b). Similarly, muscle glucose

uptake is attenuated in muscle-specific Rac1 knockout mice in response to *ex vivo* contractions and stretch (Sylow *et al.*, 2013b; Sylow *et al.*, 2015; Sylow *et al.*, 2017b), and *in vivo* exercise (Sylow *et al.*, 2016; Sylow *et al.*, 2017b). In experimental work by another group, both Rac1 inhibition and siRNA-targeted knockdown of Rac1 was reported to attenuate GLUT4 translocation in response to electrical pulse stimulation in C2C12 cells (Hu *et al.*, 2018).

The level of Rac1 activation and its involvement in glucose uptake appears dependent on the intensity of the contraction or exercising conditions. It has been reported that exercise-stimulated Rac1 activation in mouse soleus muscle, as measured by Rac1-GTP binding, was increased by approximately 50% and 100% during treadmill exercise at 50% and 70% of maximum running speed, respectively (Sylow *et al.*, 2013b). In Rac1 KO mice, quadriceps glucose uptake during treadmill running was reduced to a greater extent when exercising at 85% maximal running capacity (~50% reduction) compared to 65% maximal running capacity (~30% reduction) (Sylow *et al.*, 2016).

As mentioned earlier in this review, Rac1 has been suggested to be linked to mechanical-dependent signals, but not in response to signals arising from metabolic disturbance. Indeed, stretch-stimulated glucose uptake is attenuated in the presence of chemical Rac1 inhibition and Rac1 knockout models (Sylow *et al.*, 2015). However, in experimental models where muscles are electrically stimulated to contract but force production is prevented, Rac1 does not appear to be involved in stimulating glucose uptake

(Sylow *et al.*, 2015). As such, other signalling proteins, known to be activated by mechanical stimulus should be examined for their potential involvement in Rac1 signalling.

2.3.7.1. Rac1 downstream signalling

Studies in both cell culture and mature muscle preparations provide some evidence that regulation of glucose uptake by Rac1 could occur via modulating the actin cytoskeleton. Activation of Rac1 leads to polymerization of the actin cytoskeleton into cortical structures (Ridley, 2006). The precise role of these actin structures in this regard is not clear, but could serve to facilitate GLUT4 translocation by serving as 'tracks' for molecular motor proteins to traffic GLUT4 vesicles (Bose *et al.*, 2002; Toyoda *et al.*, 2011; Boguslavsky *et al.*, 2012), or play a role in signal transduction by providing a scaffold for the spatial organisation of signalling proteins (Khayat *et al.*, 2000). In cell culture studies, pharmacological disruption of the actin cytoskeleton has been shown to block the increase in glucose uptake in response to insulin (Omata *et al.*, 2000; Bose *et al.*, 2001; Kanzaki *et al.*, 2001). Likewise, treatment of isolated EDL and soleus mouse muscles with inhibitors that disrupt the proper functioning of the cytoskeleton also attenuate glucose uptake in response to contraction and stretch (Sylow *et al.*, 2013b; Sylow *et al.*, 2015). However, due to the difficulty in measuring actin dynamics in mature muscle preparations compared to cells, studies have not yet demonstrated that Rac1 KO mice or Rac1 inhibitors result in perturbed cytoskeletal regulation during contractions or exercise. Therefore, directly linking Rac1 singling towards the actin cytoskeleton and glucose uptake in mature muscle is yet to be fully established.

Muscle contractions have been reported to activate p21-activated kinase (PAK) which is downstream of Rac1. PAK1 and PAK2 are expressed in skeletal muscle and phosphorylation of PAK in response to muscle contraction has been reported to correspond to Rac1-GTP binding and activation (Sylow *et al.*, 2013b). Corroborating with this, PAK signalling in muscles from Rac1 knockout mice is attenuated during contraction (Sylow *et al.*, 2013b). Because measuring Rac1 activation by Rac1-GTP binding assay is difficult in mature muscle (L. Sylow, University of Copenhagen, *personal communication*), phosphorylation of PAK has often been used as a surrogate measure of Rac1 activity in mature muscle models. Blockade of PAK1 in muscle cells and mouse muscle by chemical inhibitor or PAK1 knockout has been reported to reduce GLUT4 translocation in response to insulin stimulation (Wang *et al.*, 2011; Tunduguru *et al.*, 2014) suggesting that PAK is required in the Rac1 signalling pathway. However, a recent series of extensive experiments showed that despite attenuation of contraction-stimulated glucose uptake in soleus and EDL muscles treated with the PAK inhibitor IPA-3, PAK1 knockout mice exhibit normal contraction-stimulated glucose uptake (Moller *et al.*, 2019). These findings highlight the potential challenges of signalling redundancy when attempting to examine the role of target proteins using knockout mouse models. Therefore, although PAK phosphorylation may indicate Rac1 activity, and is useful in that sense, its involvement in glucose uptake is not clear.

Blockade of Rac1 signalling in muscle during contraction or exercise, by chemical inhibitors or Rac1 knockout does not affect signalling of AMPK, p38, and TBC1D1/4 (Sylow

et al., 2013b; Sylow *et al.*, 2015; Sylow *et al.*, 2017b) indicating that these proteins, although involved in muscle glucose uptake during contraction, do not act downstream of Rac1.

There is some evidence that nNOSμ is a downstream target of Rac1. nNOSμ is expressed in skeletal muscle and both nNOSμ and Rac1 can be targeted to the dystrophin glycoprotein complex (Brenman *et al.*, 1995; Oak *et al.*, 2003) and it has been reported that Rac1 activation stimulates NOS activity in human aortic endothelial cells (HAEC), whereas supressing Rac1 activation results in a diminished NOS activity (Selvakumar *et al.*, 2008). However, the targeting of NOS by Rac1 has not been observed in muscle cells, and experimental work should be carried out to determine if this is the case. On the other hand, it appears that Rac1 may act downstream of NOS (Godfrey & Schwarte, 2010). The link between Rac1 and NOS will be discussed further within this review (see section 2.4).

2.3.7.2. Regulation of Rac1 signalling by RhoGDI

RhoGDI is a cytosolic protein considered to primarily function as a 'negative regulator' of Rho GTPase activity. RhoGDI's sequester Rho GTPases in a cytosolic complex, modulating their activity by preventing nucleotide dissociation (GDP or GTP) and interaction effector targets (Garcia-Mata *et al.*, 2011). On the other hand, complexation of Rho GTPase with RhoGDI is also understood to protect Rho GTPases from proteasome-dependent degradation (Boulter & Garcia-Mata, 2012) therefore serving a role in the stability of RhoGTPase content. GDI displacement factors (GDF's) promote the release of Rho GTPases

from their inhibitory complex with RhoGDI (Kuhlmann *et al.,* 2016) allowing subsequent activation and interaction with effector targets.

There is building rationale that RhoGDI may indeed negatively regulate glucose uptake by reducing Rac1 activity. In cell culture experiments, the induction of RhoGDI was shown to block insulin-stimulated actin cytoskeleton reorganisation (Nishiyama *et al.,* 1994), a cell process which involves Rac1 activation. In that study, the inhibitory effect of RhoGDI induction on insulin-stimulated actin reorganisation was reversed by the presence of GTP-bound Rac1 (Nishiyama *et al.,* 1994). While three RhoGDI isoforms have been identified (RhoGDIα, RhoGDIβ, and RhoGDIγ) targeting a number of Rho GTPase family members (Dovas & Couchman, 2005), RhoGDIα is known to be expressed in skeletal muscle and also target Rac1 (Zalcman *et al.*, 1996; DerMardirossian *et al.*, 2004; Dovas & Couchman, 2005). Preliminary findings from Dr Lykke Sylow's group in Denmark report that RhoGDIα negatively regulates Rac1 signalling and glucose uptake in muscle. In these experiments, transfection of siRNA targeting RhoGDIα in L6 muscle cells to reduce RhoGDIα content resulted in an increase in basal- and insulin-stimulated Rac1 activity concomitant with an increased basal- and insulin-stimulated GLUT4 translocation (Moller *et al.,* 2017). In further work, mice over-expressing muscle RhoGDIα were found to have impaired whole-body glucose tolerance and attenuated insulin-stimulated glucose uptake (Moller *et al.*, 2017), suggestive of impaired Rac1 signalling. However, the role of RhoGDIα in regulating contraction-stimulated glucose uptake has not yet been explored.

Interestingly, evidence of a Rac1 downstream effector feedback loop exists. Phosphorylation of RhoGDI by the Rac1 downstream kinase PAK1 has been reported to promote the release of Rac1 from complex with RhoGDI in HEK293T cells and HeLa cells (DerMardirossian *et al.*, 2004), and treatment of cells with the PAK inhibitor IPA-3 leads to reduced Rac1 activation (Byrne *et al.*, 2016). However, such a PAK-Rac1 feedback loop may not be relevant to mature muscle considering that PAK KO mice are reported to have normal contraction-stimulated glucose uptake (Moller *et al.*, 2019), and further work is required to better understand the complex regulation of Rac1 signalling in muscle.

2.3.8. Nitric Oxide (NO) and NO synthase (NOS)

2.3.8.1. Basal glucose uptake

Balon & Nadler (1994) first reported in isolated rat muscle preparations that NO production and basal glucose uptake are reduced in the presence of the NOS inhibitor L-NMMA, suggesting that NO regulates basal glucose uptake. This finding was supported by some studies (Balon & Nadler, 1997; Stephens *et al.*, 2004), whereas other studies found that the presence of NOS inhibitors did not lower basal muscle glucose uptake (Higaki *et al.*, 2001; Merry *et al.*, 2010b; Hong *et al.*, 2015b). nNOSμ is the main source of NO production in skeletal muscle (Lau *et al.*, 2000) and nNOSμ KO mice, which have little detectable NOS activity have similar levels of basal glucose uptake to that of wildtype mice (Hong *et al.*, 2015b). Likewise, in humans, one study reports that arterial infusion of L-NMMA did not

alter leg glucose uptake at rest compared to a control group (saline infusion) (Heinonen *et al.*, 2013). Therefore, the majority of studies suggest that NO does not play a role in regulating basal glucose uptake in skeletal muscle.

NO-releasing compounds have been shown to enhance basal glucose uptake in isolated rodent muscle (Balon & Nadler, 1997; Etgen *et al.*, 1997; Young *et al.*, 1997; Young & Leighton, 1998b; Higaki *et al.*, 2001; Merry *et al.*, 2010b), and in human muscle strips (Deshmukh *et al.*, 2010). Similarly, infusion of the NO donor sodium nitroprusside (SNP) into the femoral artery of humans increased leg glucose uptake at rest (Durham *et al.*, 2003). It is important to note, however, that NO donors likely exert different signalling to that of NOS-derived NO signalling *in vivo* at physiological levels. Indeed, in cell culture experiments, exogenous NO from NO donors was shown to result in a markedly different spatial distribution and signalling to that of endogenously produced NO (Iwakiri *et al.*, 2006). Therefore, findings from NO donor studies are unlikely to fully represent physiological NO signalling and should be interpreted with caution.

2.3.8.2. Contraction- and exercise-stimulated glucose uptake

Skeletal muscle NOS activity is markedly increased by exercise and contractions in rodents (Balon & Nadler, 1997; Roberts *et al.*, 1999; Ross *et al.*, 2007; Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b; Hong *et al.*, 2016) and exercise in humans (Linden *et al.*, 2011). These findings are supported by *in vitro* cell culture studies using NO-sensitive fluorescent probes to examine NO production in real-time during contractions (Silveira *et*

al., 2003; Pattwell *et al.*, 2004; Pye *et al.*, 2007). In addition, there is evidence that mechanical strain in the form of passive stretching is sufficient to stimulate NO production in cells and muscle fibres (Tidball *et al.*, 1998; Zhang *et al.*, 2004; Wozniak & Anderson, 2009). The role of NOS activity in regulating muscle glucose uptake during contraction or exercise has been studied in a number of human and rodent models. However, controversy exists as to whether NOS is an essential regulatory factor of muscle glucose uptake during exercise or contraction and will be discussed in detail in the following sections.

Human studies

Human studies have largely found that NOS inhibitors attenuate the normal increase in muscle glucose uptake during exercise (Table 2.1). Our group demonstrated that femoral arterial infusion of the NOS inhibitor L-NMMA attenuated leg glucose uptake by ~40-50% during cycling exercise in young, healthy participants, and this effect tended to be reversed by the co-administration of the NOS substrate L-arginine (Bradley *et al.*, 1999). However, in that study, L-NMMA was initially infused at a dose of 0.4 mg per kg body mass per min before the infusion dose was lowered by half. The observed reversal of the L-NMMA effect by the co-administration of L-arginine may have actually reflected the lower L-NMMA dose (Bradley *et al.*, 1999). Importantly, in a follow-up study, it was found that L-NMMA attenuated leg glucose uptake during cycling exercise in healthy control subjects (~35%) and that this effect was greater (~70%) in people with type 2 diabetes (Kingwell *et al.*, 2002). In that study, 0.4 mg per kg body mass per min was infused during the whole exercise bout and

unlike the earlier study showed no sign of lessening its effect on glucose uptake. In both studies, the effects of NOS inhibition on leg glucose uptake during exercise was independent of total blood flow. It is important to note however that total blood flow may not be reflective of changes in microvascular blood flow (Keske *et al.*, 2016), and although rodent studies suggest that NOS inhibition does not affect microvascular flow during muscle contraction (Ross *et al.*, 2007; Hong *et al.*, 2015a), no human study has confirmed that NOS inhibitors do not act on the microcirculation during exercise.

Nonetheless, the observation of increased sensitivity to L-NMMA in people with type 2 diabetes (Kingwell *et al.*, 2002) suggests that these individuals have a greater reliance on NO to achieve normal glucose uptake during exercise than healthy individuals, and highlights the potential therapeutic benefit of targeting the NO signalling pathway in the treatment of type 2 diabetes.

In support, and although not reported, arterial and venous glucose data combined with leg blood flow data in a published study by another laboratory (Mortensen *et al.*, 2007) indicated that leg glucose uptake was markedly lower (~50%) with femoral artery infusion of L-NMMA at rest and increased less (~50%) during repeated leg-extension exercise. Leg glucose uptake data, although not reported by the authors of this study, could be calculated from the presented femoral blood flow and arterial-venous glucose data. Unfortunately, as only mean data values were presented, it is not possible to conduct a statistical analysis of this data. In addition, it should be noted that although this study indicated that L-NMMA

reduced leg glucose uptake during exercise, L-NMMA was not examined in isolation, but rather combined with the cyclooxygenase inhibitor indomethacin. Nonetheless, significant reductions in resting and exercise-mediated leg blood flow (~35%) with L-NMMA and indomethacin treatment were observed in this study and therefore, it is not clear if reduced leg glucose uptake is due to intracellular factors or reduced glucose delivery available to the muscles.

In contrast, conflicting findings have been published in other studies. Heinonen et al. (2013) reported no effect of L-NMMA infusion on leg glucose uptake during leg-extension exercise. However, there are aspects of the study design which could explain the lack of L-NMMA effect on glucose uptake such as the exercise intensity, L-NMMA dose, and the order in which the control and L-NMMA trials were performed. NOS has been reported to be activated at higher, rather than, lower intensities (Silveira *et al.*, 2003; Pattwell *et al.*, 2004; Lee-Young *et al.*, 2009), and as such the leg-kicking exercise intensity (10 watts) employed by Heinonen et al. (2013) may have been insufficient to activate NOS. Indeed, as mentioned above, Mortensen et al. (2007) using a higher intensity leg kicking model (19 watts) found that glucose uptake was lower during NOS inhibition. Additionally, the L-NMMA dose reported by Heinonen et al. (2013) was approximately 65% less than the dose used in studies by our group (Bradley *et al.*, 1999; Kingwell *et al.*, 2002), and approximately 30% less than the dose used by Mortensen et al. (2007) (see Table 2.1). It is possible that this dose was not high enough to reduce muscle NOS activity during exercise. Although a significant reduction in resting blood flow was observed in that study, suggesting that the L-NMMA dose used did

indeed have some effect in reducing NOS activity, no such effect was observed during exercise. It should also be noted that in the Heinonen et al. (2013) study, participants performed the L-NMMA trial 30 minutes following the control trial (saline infusion) and as such, potential effects of NOS inhibition could have been masked by the preceding exercise bout given that activation of signalling pathways associated with glucose transport can persist for several hours following exercise (Schweitzer *et al.*, 2012; Fritzen *et al.*, 2015; Sjoberg *et al.*, 2017).

In another study, Kalliokoski and colleagues (2006) also report that NOS inhibition did not attenuate muscle glucose uptake during one-legged knee extension exercise (25 watts). Administration of the NOS inhibitor L-NAME, however, was combined with indomethacin and the combined inhibitors resulted in a significant reduction in leg blood flow during exercise making interpretation of the finding difficult. The reduction in blood flow could have resulted in an exercise-induced decrease in muscle oxygenation. In turn, this could have resulted in a compensatory increase in glucose uptake since hypoxia can stimulate intracellular factors which increase glucose uptake (Cartee *et al.*, 1991).

By using an experimental model that attempted to enhance NO production above normal exercising levels, our group demonstrated that infusion of the NOS substrate L-arginine increased leg glucose uptake above control (saline infusion) values during prolonged exercise (McConell *et al.*, 2006). However, in a follow-up, study the greater glucose uptake with L-arginine infusion was accompanied with a significant elevation of plasma insulin

concentrations while no elevation in skeletal muscle NOS activity was observed (Linden *et al.*, 2011). In the initial study (McConell *et al.*, 2006), there was a tendency for increased plasma insulin levels, however, a significantly increased plasma insulin may have been missed in that study because blood samples were drawn at 15 min intervals compared to 5 min intervals in the follow-up study (Linden *et al.*, 2011). Therefore, the increased glucose disposal was likely attributed to elevated plasma insulin concentrations, and not by a skeletal muscle NOS-dependent mechanism.

Table 2.1 Overview of NOS inhibitor studies during exercise in humans

Study	Subject characteristics	Exercise protocol	NO measure	Inhibitor	Result
Bradley et al. (1999)	Healthy, normally active but not exercise-trained males (aged 23-35 years)	30 min supine cycling at 60% $\dot{V}_{O_2}$ peak	Not measured	L-NMMA (0.4 mg · kg^{-1} body mass · min^{-1}), infused into femoral artery from 10-15 min after exercise start and then (0.2 mg · kg^{-1} body mass · min^{-1}) thereafter.	↓ ~50%
Kingwell et al. (2002)	Healthy, normally active but not exercise-trained males (aged 46±5 years)	25 min supine cycling at 60% $\dot{V}_{O_2}$ peak	Not measured	L-NMMA (0.4 mg · kg^{-1} body mass · min^{-1}), infused into femoral artery 10min after start of exercise	↓ ~35%
Kingwell et al. (2002)	Type 2 diabetic, normally active but not exercise-trained males (aged 48±5 years)	25 min supine cycling at 60% $\dot{V}_{O_2}$ peak	Not measured	L-NMMA (0.4 mg · kg^{-1} body mass · min^{-1}), infused into femoral artery from 10min of exercise	↓ ~75%

Table 2.1 Overview of NOS inhibitor studies during exercise in humans (continued)

Study	Subject characteristics	Exercise protocol	NO measure	Inhibitor	Result
Mortensen et al. (2007) #	Healthy, recreationally active males (aged 24±4 years)	5 min one-legged knee extensions, 19±1 W (corresponding to 20% of maximal output)	Not measured	L-NMMA (reported as 1.0 mg · min^{-1} kg leg mass^{-1}) corresponding to 0.2 mg · kg^{-1} body mass · min^{-1}, infused into the femoral artery 5 min before and during exercise. L-NMMA co-infused with Indomethacin (50 µg· min^{-1} · kg leg mass^{-1})	↓ ~50% at rest and during exercise #
Heinonen et al. (2013)	Healthy, untrained males (aged 26±2 years)	7.5 min one-legged knee extensions, ~10 W	Not measured	L-NMMA (1.0 mg · min^{-1} · kg leg mass^{-1}), estimated as 1.2 – 1.4 mg · kg^{-1} body mass · min^{-1} infused into the femoral artery 10 min before and during exercise.	No effect of L-NMMA
Kalliokoski et al. (2006)	Healthy males (aged 24±5 years)	15 min one-legged knee extensions, 25 W	Not measured	10 mg/mL L-NAME solution administered directly into vastus lateralis muscle via microdialysis probe at a rate of 2 µL/min, 1 hour before onset of exercise. L-NMMA co-infused with 100 mg/mL Indomethacin	No effect of L-NAME

L-NMMA, N^G-Monomethyl-L-arginine. L-NAME, N^G-Nitro-L-arginine methyl ester. Result column represents the change in glucose uptake. # Glucose uptake was not calculated by the authors but available to be calculated from the presented data. Because only means were available it was not possible to conduct. statistical analysis on this study.

Rodent studies

Evidence from rodent studies regarding the role of NO in the regulation contraction- and exercise-mediated glucose uptake are conflicting. NOS inhibition has been reported to attenuate contraction-mediated glucose uptake in *ex vivo* and *in situ* models in some (Balon & Nadler, 1997; Stephens *et al.*, 2004; Ross *et al.*, 2007; Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b) but not all (Etgen *et al.*, 1997; Higaki *et al.*, 2001; Inyard *et al.*, 2007; Hong *et al.*, 2015a) studies. Similarly, conclusions from acute exercise studies are unclear with one study reporting that several days ingestion of the NOS inhibitor L-NAME attenuated exercise-mediated glucose uptake (Roberts *et al.*, 1997), while other studies using a similar approach oppose this finding (Higaki *et al.*, 2001; Rottman *et al.*, 2002). It is difficult to make direct comparisons between studies given numerous methodological differences such as the timing of glucose uptake measurement, rodent model, strain of rodent, timing of NOS inhibition, muscles examined, and exercise/contraction protocols. The following section will discuss how these methodological factors may contribute to the differences in findings and the studies are summarised in Table 2.2 - Table 2.4.

Table 2.2 Overview of NOS inhibitor studies in *ex vivo* rodent models

Study	Species/Strain/Muscles	Contraction/ Exercise protocol	Contraction increase in NO?	Glucose uptake measurement	Inhibitor	Result
Etgen et al. (1997)	Rat, Sprague-Dawley, epitrochlearis	120 contractions/ min, 200 ms train, 2 x 10 min contraction periods separated by 1 min rest	cGMP content ↑~130% (not sig.)	≥ 10 min post contraction	L-NMMA, 100 µM	No effect
Higaki et al. (2001)	Rat, Sprague-Dawley, soleus, EDL	2 contractions/ min, 10 s train, 10 min	Not measured	Immediately following contraction	L-NMMA, 100 µM	No effect
Stephens et al. (2004)	Rat, Sprague-Dawley, epitrochlearis	1 contraction/ min, 10 s train, 10 min	Not measured	≥ 10 min post contraction	L-NMMA, 100 µM	↓ ~60%
Merry et al. (2010c)	Mouse, AMPK DN and WT, EDL	12 contractions/ min, 350 ms train, 10 min	NOS activity ↑~100%	During final 5 min of contraction and 5 min of recovery	L-NMMA, 100 µM	↓ ~40%
Merry et al. (2010c)	Mouse, AMPK DN and WT, Soleus	12 contractions/ min, 600 ms train, 10 min	Not measured	During final 5 min of contraction and 5 min of recovery	L-NMMA, 100 µM	No effect
Merry et al. (2010b)	Mouse, C57BL/6, EDL	25 contractions/ min, 600 ms train, 10 min	NOS activity ↑~35%	During final 5 min of contraction and 5 min of recovery	L-NMMA, 100 µM	↓ ~50%
Hong et al. (2015b)	Mouse, nNOS KO, EDL	12 contractions/ min, 350 ms train, 10 min	No change in NOS activity	During final 5 min of contraction	L-NMMA, 100 µM	↓ ~20%
Hong et al. (2015b)	Mouse, nNOS WT, EDL	12 contractions/ min, 350 ms train, 10 min	NOS activity ↑~35%	During final 5 min of contraction	L-NMMA, 100 µM	↓ ~20%

Table 2.3 Overview of NOS inhibitor studies in *in situ* rodent models

Study	Species/ Strain/Muscles	Contraction/ Exercise protocol	Contraction increase in NO?	Glucose uptake measurement	Inhibitor	Result
Balon et al. (1997)	Rat, Sprague-Dawley, EDL	2 x 5 min, 500 ms train/s, 6 - 8 V	Not measured	Muscle dissected, glucose uptake measured ≥ 70 min post contraction	L-NMMA, 1 µM	↓ ~90%
Higaki et al. (2001)	Rat, Sprague-Dawley, EDL	2 x 5 min, 500 ms train/s, 100 Hz 3 - 10 V	Not measured	Muscle dissected, glucose uptake measured ≥ 20 min post contraction	L-NMMA, 100 µM	No effect
Inyard et al. (2007)	Rat, Sprague-Dawley, hindlimb muscles	0.05 – 2 Hz, 0.5 ms, 2 V, 10 min at each freq.	Not measured	During exercise, arterial/venous glucose x femoral blood-flow	L-NAME, 50 µg/min/kg	↓ ~50% (not sig.)
Ross et al. (2007)	Rat, hooded Wistar, hindlimb muscles	2 Hz 0.1 ms, 35 V, 30 min	NOS activity ↑~35%	During final 10 min of contraction	L-NAME, 5 µM	↓ ~35%
Hong et al. (2015a)	Rat, Sprague-Dawley, hindlimb muscles	2 Hz 0.1 ms, 35 V, 30 min	No change in NOS activity	During final 10 min of contraction	L-NAME, 5 µM	No effect
Hong et al. (2015a)	Rat, Sprague-Dawley (T2D model#), hindlimb muscles	2 Hz 0.1 ms, 35 V, 30 min	No change in NOS activity	During final 10 min of contraction	L-NAME, 5 µM	No effect

Table 2.4 Overview of NOS inhibitor studies in *in vivo* rodent models

Study	Species/Strain/Muscles	Contraction/ Exercise protocol	Increase in NO with exercise?	Glucose uptake measurement	Inhibitor	Result
Roberts et al. (1997)	Rat, Sprague-Dawley, hindlimb muscles	Treadmill running, 45 min, 15 - 20% grade, 1.3 - 1.9 km/h	Not measured	Muscle frozen immediately following exercise, sarcolemma vesicle glucose transport measured	L-NAME, 1mg/ml drinking water for 2 days.	↓ ~100%
Higaki et al. (2001)	Rat, Sprague-Dawley, soleus	Treadmill running, 60 min, 10% grade, 1.1 km/h	Not measured	Muscles dissected immediately following exercise, glucose uptake measured ≥ 20 min post exercise	L-NAME, 1mg/ml drinking water for 2 days	No effect
Rottman et al. (2002)	Mouse, C57BL/6, hindlimb muscles	Treadmill running, 30 min, 0% grade, 1 km/h	Not measured	During final 25 min of exercise	L-NAME, 1mg/ml drinking water for 3 days	No effect

EDL, extensor digitorum longus. WT, wildtype littermate. L-NMMA, N^G-Monomethyl-L-arginine. L-NAME, N^G-Nitro-L-arginine methyl ester. Result column represents the change in glucose uptake. # T2D model, high-fat diet and streptozotocin-induced. Not sig., Not significant.

Timing considerations of glucose uptake measurement and NOS inhibition

NOS inhibition studies have either examined glucose uptake during muscle contractions or in the period following contraction. Mechanisms that regulate glucose uptake during contraction are likely better reflected by the examination of glucose uptake during the contractile period. When glucose uptake was measured during contraction, some studies (Ross *et al.*, 2007; Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b), but not all studies (Rottman *et al.*, 2002; Inyard *et al.*, 2007; Hong *et al.*, 2015a) report that NOS inhibition attenuates glucose uptake during contractions or exercise. It should be noted that in the study by Inyard et al. (2007) NOS inhibition appeared to cause a reduction in glucose uptake during contraction (more than 50%), however, this was not reported a statistically significant difference. The statistical analysis of that experiment was run over many different frequencies of contraction (from low to high), and as one would expect only the higher frequency showed alterations in response to NOS inhibition. Conflicting findings between our studies in rats (Ross *et al.*, 2007; Hong *et al.*, 2015a) despite measuring glucose uptake during contractions possibly reflect differences in strains used and is discussed in more detail later within this section (see *Rodent strain differences*).

Findings from studies that measured glucose uptake following contractions are also equivocal, with some studies indicating that glucose uptake is attenuated by NOS inhibition (Balon & Nadler, 1997; Roberts *et al.*, 1997; Stephens *et al.*, 2004), while others report no effect (Etgen *et al.*, 1997; Higaki *et al.*, 2001). In the study by Etgen et al (1997), it is important to note that NOS did not appear to be activated by contractions as the levels of cGMP, a molecule activated by NO, was not increased with contractions. Nonetheless, these later studies

may be more relevant to mechanisms relating to the post-contraction period and not *during* contraction.

The timing and duration in which NOS inhibitors are applied is another methodological factor worth considering. In two studies, EDL muscles were excised from rats following *in situ* contractions and then incubated in the presence of L-NMMA prior to measurement of glucose uptake (Balon & Nadler, 1997; Higaki *et al.*, 2001). Interestingly, despite the lack of NOS inhibition *during* contraction, one study (Balon & Nadler, 1997) reported that the post-contraction NOS inhibition resulted in a complete blockade of contraction stimulated glucose uptake whereas another study failed to demonstrate this effect (Higaki *et al.*, 2001). Differences in L-NMMA incubation times following contraction (70 min vs 20 min) between these studies could explain this discrepancy, where longer duration of NOS inhibition is required to influence glucose uptake. However, given that reports that NO production returns to baseline levels briefly after the contractile activity has ceased (< 5 min) (Tidball *et al.*, 1998; Pye *et al.*, 2007), it is worth considering whether the effects of L-NMMA on glucose uptake are due to off-target effects. Nonetheless, the application of NOS inhibitors during the post-contraction period is not relevant to NOS activity during contractions.

Muscles examined

Muscle fibre type differences in NOS signalling likely exist and therefore the muscle examined in NOS studies should be considered. Indeed, one study reported that levels of the NO downstream signalling intermediate cGMP were increased in EDL but not soleus muscles following contractions (Lau *et al.*, 2000) and our group has demonstrated that NOS inhibition attenuates *ex vivo* contraction-stimulated glucose uptake in EDL but not soleus muscles in mice

(Merry *et al.*, 2010c). The predominance of NOS signalling in glycolytic muscles compared to oxidative muscles could, therefore, explain why NOS inhibition failed to attenuate contraction-stimulated glucose uptake in soleus muscles of rats in another study (Higaki *et al.*, 2001). However, there are conflicting reports where studies have used identical glycolytic muscles. Indeed, a study by our group reported an ~70% attenuation of the increase in glucose uptake with L-NMMA in the glycolytic epitrochlearis muscles from rats (Stephens *et al.*, 2004), while earlier a study from another laboratory using the same muscle type found no effect of L-NMMA (Etgen *et al.*, 1997). Similarly, it was reported that L-NMMA attenuated *ex vivo* contraction-stimulated glucose uptake in rat EDL muscles in one study (Balon & Nadler, 1997), while this finding was opposed in another study using a similar rat EDL muscle and contraction model (Higaki *et al.*, 2001). Therefore, although fibre-type differences are potentially involved, several contradictory results from different studies using similar or identical muscles indicate that other experimental factors need to be considered.

Muscle stimulation intensity and NOS activation

Measurement of NO production or NOS activation has been an often-overlooked consideration of the NOS inhibitor studies published to date. Studies indicate that NOS activation is likely dependent on the intensity of contractile activity. Indeed, while muscle NOS activity in mice was found to not increase following low-intensity treadmill running (45% max speed), running at higher-intensities (70% max speed) resulted in a significant increase in NOS activity (Lee-Young *et al.*, 2009). In agreement, NO release in contracting muscle cells was higher at 50 Hz stimulations compared to 1 Hz (Pattwell *et al.*, 2004). Therefore, where studies have not reported a measure of NOS activity, it is difficult to determine whether a lack NOS

inhibitor effect on muscle glucose uptake during exercise/contractions is due to the exercise/contraction protocol lacking the intensity to stimulate NOS. Indeed, we have previously shown that identical *in situ* electrical stimulation protocols failed to activate NOS in one strain of rat (Hong *et al.*, 2015a) while significantly activating muscle NOS activity in another strain (Ross *et al.*, 2007). The failure of the stimulation protocol to activate NOS was consistent with a lack of NOS inhibitor effect on glucose uptake (Hong *et al.*, 2015a), while in the study where NOS activation was observed, the presence of the NOS inhibitor attenuation contraction-stimulated glucose uptake (Ross *et al.*, 2007). In another study in rats, *in situ* contractions over a range of stimulation frequencies indicated that L-NAME did not affect contraction-stimulated glucose uptake at lower stimulation frequencies (0.1 – 0.5 Hz), however, at a higher contraction frequency (2 Hz), L-NAME appeared to attenuate glucose uptake by approximately 50% (Inyard *et al.*, 2007). Despite this, the difference in contraction-stimulated glucose uptake between L-NAME and saline control muscles was not reported to be significantly different. In an earlier study in mice, contraction-stimulated glucose uptake was not altered by L-NMMA, however, the contraction protocol was likely insufficient to activate NOS given no significant production of the NO downstream intermediate cGMP was observed (Etgen *et al.*, 1997). Therefore, where measures of NO production or NOS activity have not been conducted by studies, interpretation should not proceed without caution.

Rodent strain differences

Studies from our group have reported that muscle glucose uptake during *in situ* contractions was attenuated by local infusion of L-NAME in hooded Wistar rats (Ross *et al.*, 2007) but not when in Sprague-Dawley rats (Hong *et al.*, 2015a). While this discrepancy could

point to a difference in rat strain used, other studies have demonstrated that contraction- or exercise-stimulated skeletal muscle glucose uptake is reduced by NOS inhibition in Sprague-Dawley rats (Balon & Nadler, 1997; Roberts *et al.*, 1997; Stephens *et al.*, 2004). Nonetheless, it remains a possibility that strain differences or even sub-strain differences confound NOS inhibitor studies. Indeed, there is evidence that the hypertensive response to chronic L-NAME treatment is significantly different in Sprague-Dawley rats maintained in separate colonies for several generations (Pollock & Rekito, 1998; Buhimschi *et al.*, 2001). However, the observation that NOS regulates glucose uptake during contraction/exercise in some strains and not others remains to be directly examined within the same study.

NOS isoform knockout mouse models

The investigation of NO-mediated glucose uptake in rodents has mostly relied on the use of pharmacological inhibitors. However, since inhibitors can have off-target effects, genetic mouse models provide an important strategy to probe the role of NO (Lee-Young *et al.*, 2010; Hong *et al.*, 2015b; Hong *et al.*, 2016). nNOSμ is the predominant source of NO in contracting rodent skeletal muscle (Lau *et al.*, 2000; Hong *et al.*, 2015b). Skeletal muscle from mice lacking nNOSμ have almost no basal NOS activity and there is no detectable increase in NOS activity with contractions or exercise (Hong *et al.*, 2015b; Hong *et al.*, 2016). In agreement, contraction was shown to elevate the content of the downstream NO intermediate cGMP in EDL muscle from wildtype, but not nNOSμ KO mice, whereas the muscles lacking eNOS had normal elevations of cGMP with contraction (Lau *et al.*, 2000). Surprisingly, however, compared with controls, skeletal muscle glucose uptake was normal in nNOSμ KO mice during *ex vivo* contractions (Hong *et al.*, 2015b) and significantly higher than wild type

mice during *in vivo* exercise (Hong *et al.*, 2016). These findings suggest a dissociation between NOS activity and glucose uptake. Surprisingly, L-NMMA during *ex vivo* contraction attenuated contraction-stimulated glucose uptake in nNOSμ KO mice to a similar extent to wildtype littermates (~20%) (Hong *et al.*, 2015b), suggesting that a different NOS isoform could be regulating glucose uptake. eNOS is also expressed in mouse EDL muscle and was the only NOS isoform detected in nNOSμ KO skeletal muscle in studies described above (Hong *et al.*, 2015b; Hong *et al.*, 2016). In a previous study, however, it was found that like nNOS KO mice, skeletal muscle glucose uptake was surprisingly elevated during treadmill running in mice lacking eNOS (Lee-Young *et al.*, 2010). However, the loss of eNOS in the endothelium could have resulted in impairments to blood flow during exercise and perhaps hypoxia, which can stimulate glucose uptake (Cartee *et al.*, 1991), and therefore further investigations should be conducted in an isolated muscle preparation where the influence of blood flow is removed.

nNOSμ KO mice are generated by the targeted deletion of exon 2 of the nNOS gene (Huang *et al.*, 1993). However, nNOSβ is not encoded by exon 2, and should therefore be maintained in these mice (Percival *et al.*, 2010). Considering that mice lacking the μ isoform maintain normal contraction-mediated glucose uptake (Hong *et al.*, 2015b), it is tempting to postulate that nNOSβ is the putative NOS isoform regulating skeletal muscle glucose. However, although nNOSβ expression is well documented in skeletal muscle of wild-type mice, there is controversy as to whether this isoform is maintained in skeletal muscles of nNOSμ KO mice (Rothe *et al.*, 2005; Percival *et al.*, 2010; Baum *et al.*, 2013; Hong *et al.*, 2015b; Hong *et al.*, 2016). In our lab, we have not been able to detect the nNOSβ isoform in nNOSμ KO mice using immunoblotting techniques (Hong *et al.*, 2015b; Hong *et al.*, 2016). In

agreement with the lack of detectable nNOSβ with immunoblots by our group, RT-PCR analysis performed by Baum et al (2013) suggest that the nNOSβ splice variant is not expressed in muscles from nNOSμ KO mice. In contrast, studies which have used immunohistochemistry staining techniques have reported that expression of the β isoform is preserved in nNOSμ KO mice (Rothe *et al.*, 2005; Percival *et al.*, 2010). Therefore, the existence and function of the β isoform in nNOSμ KO mice requires clarification. Nonetheless, a different nNOS KO model generated by the targeted deletion of exon 6 of the nNOS gene does exist (Gyurko *et al.*, 2002). Also known as the KN2 mouse, these mice are reported to lack all nNOS splice variants and despite the disagreement as to whether the β isoform exists in nNOSμ KO mice, the KN2 mouse does appear to display a phenotypic difference to mice lacking only nNOSμ (Percival *et al.*, 2010). Contraction experiments in these mice would help elucidate the role of nNOS in regulating skeletal muscle glucose uptake, although such experiments could prove challenging as these mice appear to have disrupted cytoskeletal architecture and are susceptible to exercise-induced fatigue compared to wildtype mice (Percival *et al.*, 2010).

Although NO is known as a potent vasodilator, the reported effects of NOS inhibition on glucose uptake during exercise and contraction are probably due to intramuscular factors. Indeed, NOS inhibition did not reduce total leg blood flow in humans during exercise in studies by our group (Bradley *et al.*, 1999; Kingwell *et al.*, 2002) or microvascular blood flow in rats during contractions *in situ* (Inyard *et al.*, 2007; Ross *et al.*, 2007). Importantly, the observation that NOS inhibition reduces glucose uptake during *ex vivo* contractions where the potential influence of blood flow is removed (Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*,

2015b) clearly indicates that the reduction in glucose uptake is the result of intrinsic factors rather than substrate delivery.

In summary, the role of NO in regulating skeletal muscle glucose uptake during contractions and exercise has been examined in several rodent models with equivocal findings. A number of methodological differences make interpretation of rodent NOS studies difficult. If NOS is indeed an essential regulator of glucose uptake, it is likely that this is not under all contraction and exercise conditions, but rather contributes within a confined range of conditions, the nature of which is not presently clear. This position is reasonable given the complexity of signalling pathways activated by muscle contraction which are likely to exhibit a high degree of redundancy. Further work is required to establish the exact role of NO in the regulation of skeletal muscle glucose uptake during contraction in rodents. This is especially the case since the evidence for a role of NO in glucose uptake during exercise in humans is quite strong.

2.3.8.3. NO downstream targets

Soluble guanylate cyclase (sGC) is the most defined target of NO, catalysing the conversion of guanosine triphosphate (GTP) to the second messenger cyclic guanosine 3',5'-monophosphate (cGMP) (Francis *et al.*, 2010). Both NO-releasing compounds and electrically-induced contractions have been shown to markedly increase cGMP above basal levels in isolated rodent muscle (Lau *et al.*, 1998; Young & Leighton, 1998a; Lau *et al.*, 2000). In a series of studies, Young and Leighton (1997; 1998a, b) reported that basal glucose uptake was increased in isolated rat muscle by NO donors, and this occurred via a sGC/cGMP-dependent

mechanism. However, contraction-stimulated glucose uptake was shown to be unaffected by inhibition of this pathway (Merry *et al.*, 2010b), therefore indicating that during contraction, NO mediates glucose uptake via cGMP-independent mechanisms. Therefore, cGMP may play a role in basal NO-stimulated muscle glucose uptake but not during exercise.

S-nitrosylation represents a reversible post-translational protein modification analogous to phosphorylation and is emerging as an important mechanism of cGMP-independent NO signal transduction (Hess *et al.*, 2005). Increased S-nitrosothiol protein content has been reported following acute exercise *in vivo* in rodents (Nogueira *et al.*, 2009). Work from our group (Merry *et al.*, 2010b) attempted to examine whether NO mediates contraction-stimulated glucose uptake via protein S-nitrosylation by exposing contracting muscles to white light, which is suggested to break S-nitrosylated bonds (Borutaite *et al.*, 2000). Contraction-stimulated glucose uptake was normal in the presence of white light (Merry *et al.*, 2010b), suggesting that an S-nitrosylation mechanism is not involved. It should be noted, however, that in this study protein S-nitrosylation was not examined so it cannot be concluded that any changes in S-nitrosothiol content occurred. At present, S-nitrosylation of specific proteins with respect to muscle glucose uptake during contraction is yet to be established.

S-glutathionylation is another reversible post-translational modification induced by ROS and NO which involves the formation of a disulphide bond between a glutathione molecule and protein thiol (Dalle-Donne *et al.*, 2009). Findings from our group reported that the thiol-reducing agent dithiothreitol (DTT) completely blocked the increase in glucose uptake with contraction in isolated mouse muscles (Merry *et al.*, 2010b). In those experiments, it was confirmed that DTT attenuated the increase in s-glutathionylation during contraction (Merry,

2010). In other experimental work, s-glutathionylation of a protein band at ~270 kDa was enhanced in human muscle during endurance exercise (Merry *et al.*, 2010d), and in mouse EDL muscle following *ex vivo* contraction (Merry *et al.*, 2010b). S-glutathionylation of these bands was attenuated in the presence of L-NMMA or NAC during contraction in mouse muscle (Merry *et al.*, 2010b), and NAC infusion during exercise in humans (Merry *et al.*, 2010d). Further work is required to determine the physiological relevance of s-glutathionylated proteins with respect to muscle glucose uptake.

NO can interact with superoxide to form nitrating species such as nitrogen dioxide or peroxynitrite, where the incorporation of a nitro group to tyrosine residues on proteins forms a 3-nitrotyrosine residue (Radi, 2013). In a study by our group, elevated tyrosine nitration of a protein band with a molecular weight of ~37 kDa was observed with contraction, and this was blunted in the presence of L-NMMA (Merry *et al.*, 2010b), however, the protein(s) at play remain to be identified. The peroxynitrite scavenger urate was found to attenuate contraction-stimulated glucose uptake in mouse EDL muscle (Merry *et al.*, 2010b), however, this should be interpreted with caution since urate-treated muscles exhibited significantly lower peak contraction forces. In addition, passive resting-tension of muscles was significantly elevated in the presence of urate, and this was associated with a significant increase in basal glucose uptake (Merry *et al.*, 2010b). Elevations in tyrosine nitrated proteins were not detected following endurance exercise in humans (Merry *et al.*, 2010d) or *in situ* contractions in rats (Merry *et al.*, 2010a), probably the result of a lesser shift in redox status in more physiological models compared to the isolated muscle models.

Despite a number of studies having demonstrated a role for NOS in contraction-stimulated glucose uptake, the examination of the downstream protein(s) involved is somewhat limited. Studies from our group are yet to identify a definitive NOS-dependent target. AMPK is unlikely involved given that phosphorylation of AMPKα2 Thr^{172} is not affected by NOS inhibition (Ross *et al.*, 2007; Merry *et al.*, 2010b), and NOS inhibition attenuates contraction-stimulated glucose uptake in AMPKα2 KD mice to a similar extent as wild-type mice (Merry *et al.*, 2010c). In addition, deletion of nNOSμ failed to affect TBC1D1 Ser^{660} phosphorylation during treadmill exercise (Hong *et al.*, 2016), and NOS inhibition did not affect the increase of p38 phosphorylation during contraction (Merry *et al.*, 2010b). Therefore, other signalling proteins require investigation, including other TBC1D1 phosphorylation sites as well as TBC1D4 phosphorylation sites.

2.4. Does a NOS-Rac1 signalling axis regulate skeletal muscle glucose uptake?

Rac1 contains a conserved redox-sensitive motif and is susceptible to S-nitrosylation in vitro (Heo & Campbell, 2005), presenting the potential that NO directly interacts and modifies Rac1. Indeed the exposure of reactive nitrogen and oxygen species to Rac1 *in vitro* has been shown to promote GDP dissociation from Rac1 (Heo & Campbell, 2005), and incubation of C2C12 cells with a NO-donor has been reported to increase Rac1 activity (Godfrey & Schwarte, 2010). In another study using rabbit aortic endothelial cells, the administration of bradykinin, which stimulates endogenous NO production, increased Rac1 activation (Eller-Borges *et al.*, 2015). In that study, addition of the NOS inhibitor L-NAME was found to block

the increased Rac1 activation. In another study, activation of Rac1 by the NO downstream kinase PKG has been reported in HEK-293 cells (Hou *et al.*, 2004). In summary, these studies provide rationale that NO could activate Rac1. However, this should be tested in mature muscle to determine whether NO and Rac1 regulate skeletal muscle glucose uptake via the same signalling pathway.

While these studies suggest that Rac1 works downstream of NOS, a study by Selvakumar et al (2008) provides evidence that Rac1 can activate NOS. Expression of a constitutively active Rac1 (GTP-bound) in HAEC cells resulted in marked increases in NO production. When treated with a Rac1 inhibitor, basal NOS activity in HAEC and cultured neurons was reduced (Selvakumar *et al.*, 2008). Rac1 activation induces a rearrangement of the actin cytoskeleton from stress fibre-formations into cortical actin structures (Chung *et al.*, 2000). Interestingly, in HAEC cells expressing constitutively active Rac1, this shift in cytoskeletal re-organisation induced by constitutively active Rac1 was markedly blunted by L-NMMA, suggesting that Rac1 mediates actin cytoskeleton dynamics via NOS (Selvakumar *et al.*, 2008). Although in that study upregulation of Rac1 activity did not induce changes in NOS expression, another study has reported the Rac1-dependent regulation of endothelial cell eNOS at the levels of both transcription and enzymatic function (Sawada *et al.*, 2008).

The localisation of NOS could also indicate the potential of contraction-stimulated Rac1 activity to be NO-dependent. NO signalling is highly compartmentalised, largely occurring within proximity to NOS isoforms (Iwakiri *et al.*, 2006), and both nNOSμ and Rac1 localise to the mechanical-sensing dystrophin glycoprotein complex (DGC) (Brenman *et al.*, 1995; Oak *et al.*, 2003), and there is evidence that both proteins are activated by mechanical

loading (Tidball *et al.*, 1998; Sylow *et al.*, 2015). It is therefore possible that NOS and Rac1 interact to regulate muscle glucose uptake during contractions or exercise; However, such a mechanism remains untested.

2.5. Summary

Exercise, contractions and stretch all increase muscle glucose uptake via a signalling pathway that is independent of insulin-signalling. Importantly, exercise-stimulated glucose uptake is normal in people with type 2 diabetes despite impaired insulin-stimulated glucose uptake. Understanding the signalling pathways involved may contribute to the development of novel therapeutics that target this 'exercise pathway' and lower blood glucose levels in insulin-resistant individuals.

Although the exact picture is not yet clear, several signalling factors have been proposed to regulate muscle glucose uptake during stretch, contraction, and exercise including Ca^{2+}/CaMKII, AMPK, TBC1D1/4, ROS, NO, and Rac1. It is likely that the relative contribution of the factors involved is dependent on the conditions of the contracting muscle and there is likely a high degree of redundancy within the system. The role of NO in regulating skeletal muscle glucose uptake during contractions is controversial and work is required to clarify this issue. In particular, the signalling proteins downstream of NOS remain to be identified in this regard. Rac1, on the other hand, has recently emerged as a key regulator of glucose uptake in response to stretching, contraction, and exercise. There is strong rationale from cell culture models that Rac1 and NO are linked, however, this has yet to be examined in mature muscle models and in the context of glucose uptake. In addition, the regulatory protein

RhoGDI is of particular interest in Rac1-dependent glucose uptake, having been shown to negatively regulate Rac1 activity and glucose uptake in cell culture models, and this is supported by some recent unpublished findings in mice (Moller *et al.*, 2017). Overall, there is exciting potential to better understand the mechanisms underlying NO and Rac1 signalling in the regulation of muscle glucose uptake during stretch, contraction, and exercise.

2.6. Aims and Hypothesis

The general aim of this thesis was to investigate the role(s) of NO and Rac1 in the regulation of skeletal muscle glucose uptake during stretch, contraction, and exercise, with the specific aims being:

1. To examine whether NOS regulates stretch-stimulated skeletal muscle glucose uptake and whether NOS activity is linked with Rac1 signalling in this regard.
2. To examine potential interactions between NOS and Rac1 signalling in the regulation of skeletal muscle glucose uptake during contraction.
3. To examine the role of RhoGDI as an upstream regulator of Rac1 and skeletal muscle glucose uptake during exercise.

The hypotheses tested were that:

1. Activation of NOS is required for the increase in skeletal muscle glucose uptake in response to *ex vivo* mechanical loading (stretch) (Study 1).
2. NOS regulates skeletal muscle glucose uptake during *ex vivo* contraction via Rac1 signalling (Study 2).
3. Overexpression of RhoGDIα attenuates the increase in skeletal muscle glucose uptake during treadmill exercise in mice by suppressing Rac1 activity (Study 3).

CHAPTER THREE: PASSIVE STRETCH REGULATES SKELETAL MUSCLE GLUCOSE UPTAKE INDEPENDENT OF NITRIC OXIDE SYNTHASE

3.1. Introduction

Exercise as well as *ex vivo* and *in situ* muscle contractions potently stimulate the uptake of glucose into skeletal muscle via a signalling pathway that is, at least proximally, independent of the canonical insulin signalling pathway (Richter & Hargreaves, 2013). Signalling proteins that mediate glucose uptake during exercise present as an attractive therapeutic target for the treatment of Type 2 diabetes since glucose uptake and GLUT-4 translocation during contraction and exercise are mostly normal in insulin-resistant muscle (Martin *et al.*, 1995; Wojtaszewski *et al.*, 1999a; Kingwell *et al.*, 2002). However, the exact mechanisms involved remain to be fully clarified.

The transduction of mechanical stimuli into biochemical signals has long been known to regulate biological processes in skeletal muscle (Vandenburgh & Kaufman, 1979; Goldspink *et al.*, 1991; Hornberger *et al.*, 2004). Several studies have shown that mechanical loading applied to isolated rodent muscles in the form of passive stretching increases muscle glucose uptake (Ihlemann *et al.*, 1999b; Ito *et al.*, 2006; Chambers *et al.*, 2009; Jensen *et al.*, 2014; Sylow *et al.*, 2015), presumably via stimulating GLUT4 translocation (Sylow *et al.*, 2015). There is suggestion that during contractions, a mechanical signalling component is essential to fully activate the glucose transport machinery. Indeed, contraction-stimulated glucose uptake

is attenuated when tension development is impaired either by the use of myosin ATPase inhibitors or stimulating the muscle to contract at a sub-optimal length (Ihlemann *et al.,* 1999b; Blair *et al.,* 2009; Jensen *et al.,* 2014; Sylow *et al.,* 2015). However, not all studies agree with this concept (Sandstrom *et al.,* 2007), and the relative contribution requirement of 'mechanical signal' to stimulate glucose uptake during contraction may depend on the intensity of the contracting muscle (Jensen *et al.,* 2014).

Signalling pathways activated by mechanical stimuli do not appear to be linked with AMP-activated protein kinase (AMPK) since stretching does not activate AMPK and stretch-stimulated glucose uptake is normal in muscles from mice overexpressing a dominant-negative AMPK (Chambers *et al.,* 2009; Jensen *et al.,* 2014). On the other hand, passive stretching activates the cytoskeletal regulator Rac1, and stretch-stimulated glucose uptake is attenuated by Rac1 inhibitor and in Rac1 knockout mice (Sylow *et al.,* 2013b; Sylow *et al.,* 2015). However, when contracting muscles at sub-optimal resting length so as to prevent the development of tension, Rac1 inhibition failed to attenuate glucose uptake (Sylow *et al.,* 2015). Together, these studies suggest that during muscle contraction mechanical stimuli activates a distinct signalling pathway involving Rac1 that contributes to glucose uptake. As such, a passive muscle stretching model allows for this mechanical-sensitive signalling pathway, which is normally activated during contractions to be examined in isolation.

Nitric oxide synthase (NOS) activity and nitric oxide (NO) production is increased during electrical stimulations in muscle cells (Silveira *et al.,* 2003; Pattwell *et al.,* 2004), muscle contractions or exercise in rodents (Roberts *et al.,* 1999; Ross *et al.,* 2007; Merry *et al.,* 2010b; Merry *et al.,* 2010c; Hong *et al.,* 2015b; Hong *et al.,* 2016), and exercise in humans

(Linden *et al.*, 2011). Several studies have demonstrated that pharmacological inhibition of NOS attenuates the increase in skeletal muscle glucose uptake during contractile activity (Balon & Nadler, 1997; Roberts *et al.*, 1997; Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Ross *et al.*, 2007; Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b), although this is not a universal finding (Etgen *et al.*, 1997; Higaki *et al.*, 2001; Rottman *et al.*, 2002; Heinonen *et al.*, 2013; Hong *et al.*, 2015a). Conflicting findings between studies likely relates to experimental differences and are discussed in Chapter Two of this thesis. Nonetheless, further work is required to clarify the involvement of NO in regulating muscle glucose uptake.

Neuronal NOSμ (nNOSμ) is considered the predominant source of NO in contracting skeletal muscle (Lau *et al.*, 2000; Hong *et al.*, 2015b) and is largely targeted to the mechanosensing dystrophin-glycoprotein complex (DGC) at the sarcolemma (Brenman *et al.*, 1995). Acute passive stretch of both muscle cells and mature muscle has also been reported to increase NO production in muscle cells and mature muscle preparations (Tidball *et al.*, 1998; Zhang *et al.*, 2004; Wozniak & Anderson, 2009), and there is evidence that NOS is involved in the transduction of mechanical signal pathways regulating the expression of cytoskeletal proteins (Tidball *et al.*, 1999). Given the evidence that NO production is increased by stretch and NOS can participate in mechanical signalling and considering that NOS can play a role in regulating muscle glucose uptake, it is tempting to speculate that mechanical-stress (stretch) regulates glucose uptake via a NOS-dependent mechanism. Furthermore, there is *in vitro* evidence in cells that NO can modulate Rac1 activity (Heo & Campbell, 2005; Godfrey & Schwarte, 2010), placing NOS as a potential candidate protein involved in the Rac1-signalling cascade regulating stretch mediated glucose uptake (Sylow *et al.*, 2015). However, to the best

of our knowledge, no previous study has investigated the role of NO in the regulation of this pathway.

Therefore, this study aimed to determine whether acute passive stretch regulates glucose uptake via a NOS-dependent pathway. To determine this, we used two genetically modified mouse models lacking either eNOS or nNOSμ and two pharmacological NOS inhibitors which target all NOS isoforms. We hypothesized that stretch-stimulated glucose uptake in mouse EDL muscle would be attenuated by NOS inhibition and/or genetic deletion of nNOSμ.

3.2. Methods

3.2.1. Animals

All animal experimentation was conducted at the Institute for Health and Sport, Victoria University, Melbourne with the prior approval of the Victoria University Animal Ethics Committee. Animal experimentation adhered to the Australian Code of Practice for the use and care of animals for scientific purposes as described by the National Health and Medical Research Council (NHMRC) of Australia. Thirteen- to sixteen-week-old C57BL/6JArc (referred herein to C57BL/6), eNOS knockout ($eNOS^{-/-}$), and nNOSμ knockout ($nNOS\mu^{-/-}$) mice were involved in this study.

Six male mice lacking eNOS ($eNOS^{-/-}$) (Monash Animal Services, Melbourne, Australia) and eight male C57BL/6 mice (ARC, Perth, Australia) aged 14–16 weeks were used to examine the role of eNOS. The $eNOS^{-/-}$ group was generated by using $eNOS^{-/-}$ breeding pairs and therefore wildtype littermates ($eNOS^{+/+}$) were not produced. Since these mice were generated on a C57BL/6 background we chose to use C57BL/6 mice as controls.

Seven $nNOS\mu^{-/-}$ mice and six wildtype littermates ($nNOS^{+/+}$) (male and female) aged 13–15 weeks were used to examine the role of nNOSμ. $nNOS\mu^{-/-}$ (B6, 129-$NOS1^{tm1plh}$) mice were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA, stock no. 002633) (Huang *et al.*, 1993) and backcrossed onto a C57BL/6 background for at least six generations and heterozygous breeding pairs were used to obtain a colony of $nNOS^{-/-}$ and wild type littermate controls. Male C57BL/6 mice aged 13–15 weeks (ARC, Perth, Australia) were used for NOS inhibitor and NOS activity experiments. Mice were housed in standard cages and

maintained at 21°C on a 12-hour dark/light cycle with access to water and standard rodent chow ad libitum. Mice were not fasted prior to sacrifice.

3.2.2. Muscle Incubations

NOS inhibition in mice has previously been shown to attenuate the increase in contraction-stimulated glucose uptake in extensor digitorum longus (EDL) muscles, but not soleus muscles (Merry *et al.*, 2010c). In addition, electrical stimulations have been shown to elevate levels of the NO downstream intermediate cGMP in EDL but not soleus muscles (Lau *et al.*, 2000). Therefore, only EDL muscles were examined in the present study. EDL muscles were excised from anaesthetized mice (sodium pentobarbitone 70 mg/kg IP) and suspended at resting length (~2–4 mN) (Sylow *et al.*, 2015) in organ baths (MultiMyograph System; Danish Myotechnology, Aarhus, Denmark). All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Muscles were pre-incubated for 30 min in Krebs-Ringer-Henseleit buffer consisting of (mM): NaCl 118.5, $NaHCO_3$ 24.7, KCl 4.74, $MgSO_4$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.5, (pH 7.4) supplemented with 0.01% BSA, 8 mM mannitol and 2 mM sodium pyruvate. Incubation media was maintained at 30°C and continuously oxygenated with gas containing 95% O_2 and 5% CO_2 (Merry *et al.*, 2010c). Following the 30 min pre-incubation period, muscles either remained at rest or were stretched to a tension of 100–130 mN for 15 minutes (Sylow *et al.*, 2013b; Sylow *et al.*, 2015).

When the effects of the NOS inhibitors N^G-monomethyl-L-arginine (L-NMMA, 100 μM) (Roy *et al.*, 1998; Higaki *et al.*, 2001), and N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) (Roy *et al.*, 1998) were examined, these inhibitors were present during the entire 45

min incubation time. L-NMMA at this concentration has previously been shown to attenuate the increase in NOS activity by ~90% (Roy *et al.*, 1998; Higaki *et al.*, 2001; Merry *et al.*, 2010b) and contraction-stimulated glucose uptake during contraction *ex vivo* in mouse EDL by ~20-50% (Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b). L-NAME has previously been shown to exert a similar dose-dependent inhibitory effect as L-NMMA on NOS activity in skeletal muscle (Roy *et al.*, 1998). Immediately following the 45-min experimental period, muscles were quickly removed from the organ baths, washed in ice-cold Kreb's buffer, blotted dry on filter paper, snap frozen in liquid nitrogen, and stored at -80°C for future analysis.

3.2.3. Muscle Processing

NOS activity and protein signalling were determined in a separate set of muscles. To generate lysates for immunoblotting and NOS activity measurement, whole frozen EDL muscles were homogenized in ice-cold buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μl/ml protease inhibitor mixture, 50 mM sodium fluoride, and 5 mM sodium pyrophosphate] by steel beads for 2 x 30 s 30 Hz (TissueLyser, Qiagen, Valencia, CA), followed by end-over-end rotation for 30 min at 4°C. Homogenates were centrifuged at 10,000 g for 20 min at 4°C, and the supernatant collected for NOS activity measurement.

For immunoblotting, an aliquot was collected prior to the centrifugation step and diluted in sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT and 0.01% bromophenol blue) and heated at 95°C for 10 min before being subjected to

SDS-PAGE. Protein concentration was determined by the Red660 protein assay kit as per manufacturer's instructions (G Biosciences, St Louis, MO).

3.2.4. Immunoblotting

Total protein (5 µg) was separated by SDS-PAGE using stain-free gels (Bio-Rad, CA, USA) and semi-dry transferred (TransBlot Turbo system, Bio-Rad) to PVDF membranes. Prior to transfer, a stain-free image of the gel was collected to quantify total protein loading. Stain-free gel analysis indicated that no differences in protein loading were observed. Membranes were blocked for one hour at room temperature (5% skim milk in TBST), before being probed overnight at 4°C with the following primary antibodies: p-PAK1/2$^{Thr423/Thr402}$ (1:500), p-P38 MAPK$^{Thr180/Tyr182}$ (1:1000), p-AMPK^{Thr172} (1:1000), and p-CaMKII^{Thr286} (1:1000) (Cell Signaling Technology, MA, USA). The following day, membranes were incubated with HRP-secondary antibody for 1 hour at room temperature. Protein bands were visualized using Bio-Rad ChemiDoc imaging system and enhanced chemiluminescence substrate (SuperSignal West Femto, Pierce, MA, USA), and quantified using ImageLab software (Bio-Rad). Analysis of protein bands were normalized to stain-free quantification of protein loading.

3.2.5. NOS Activity Measurement

NOS activity was determined on muscle lysates in duplicate by measuring the conversion of L-[^{14}C] arginine to L-[^{14}C] citrulline (Lee-Young *et al.*, 2009; Hong *et al.*, 2015b). 15 µL of lysate (~30-60 µg total protein) was incubated in 15 µL of pre-warmed (37°C) reaction buffer containing: 50 mM Tris HCl (pH 7.5), 4 µM BH4, 100 nM calmodulin, 0.7 mM $CaCl_2$, 0.63 µM FAD, 1.15 mM NADPH, 2.5 µCi/ml [^{14}C]L-arginine, in duplicate. Duplicates

of samples were incubated in the presence or absence of 1 mM L-NAME. The concentration of L-NAME used was sufficient to block NOS activity. The assay was conducted for at 37 °C for 15 min and terminated by the addition of ice-cold buffer containing: 20 mM HEPES (pH 5.5), 2 mM EDTA and 2 mM EGTA and immediately placing the samples on ice. EDTA chelates the calcium required by eNOS and nNOS. iNOS enzymatic activity, which is calcium-independent, is terminated by the low pH (5.5) of the stop buffer. Samples were then added to spin cups containing 300 µL of equilibriated cation exchange resin followed by centrifugation at 13,000 g for 2 x 30 s separated by "rinsing" with 200 µL stop buffer. Arginine binds to the resin in the spin cups, whereas citrulline which is ionically neutral at pH 5.5 flows through the resin-containing spin cup. 500 µL of eluate was collected and transferred to scintillation vials containing 5 mL scintillation fluid (UltimaGold, Perkin Elmer, Boston, MA), thoroughly mixed, and allowed to rest for 1 hr before β-scintillation counting (Tri-Carb 2810TR, PerkinElmer, Boston, MA). NOS activity was calculated by subtracting radioactive counts of L-NAME containing samples from control sample counts and expressed as the formation [^{14}C]L-citrulline in pmol per mg protein per min.

3.2.6. Glucose Uptake Measurement

Muscle glucose uptake was calculated during the final 10 minutes of stretch or basal conditions by exchanging the incubation buffer with buffer containing 1 mM 2-deoxy-D-[1,2-^{3}H] glucose (0.128 µCi/mL) and 8 mM D-[^{14}C] mannitol (0.083 µCi/mL) (Perkin Elmer, Boston, MA) as described previously (Hong *et al.*, 2015b). This buffer also contained the NOS inhibitors when examined.

2-deoxyglucose uptake accumulation in the muscle was used as an estimate of glucose uptake (Hansen *et al.*, 1994). 2-deoxyglucose uptake is transported into the muscle cell and phosphorylated to form 2-deoxyglucose-6-phosphate which cannot undergo further oxidation (Jenkins *et al.*, 1986) Mannitol was used as a marker of extracellular glucose as it has a low affinity to glucose transporters and is not readily transported into the cell (Young *et al.*, 1986). Determination of skeletal muscle glucose uptake was performed as previously published (Merry, 2010; Merry *et al.*, 2010b; Hong *et al.*, 2015b) and described below. Frozen muscles were digested in 135 µL of 1 M NaOH for 10 min at 90°C, neutralized with 135 µL of 1 M HCl, vortex mixed, and spun at 13,000 g for 5 min. The supernatant (200 µL) was added to 4 mL of scintillation fluid (UltimaGold, Perkin Elmer, Boston, MA, USA), thoroughly mixed, and allowed to rest for 1 hr before dual isotope counting (Tri-Carb 2810TR, PerkinElmer, Boston, MA). Muscle glucose uptake was calculated by subtracting ^{14}C-mannitol as a marker of extracellular space and therefore extracellular 2-deoxyglucose, from whole-muscle ^{3}H-2-Deoxyglucose.

3.2.7. Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0 software. Glucose uptake was analysed using one (treatment)- and two (treatment and genotype)-factor ANOVA. Fisher's least significance difference test was performed if the ANOVA revealed a significant difference. Students t-test was used to compare morphological characteristics between each genotype and its relevant control, NOS activity and protein phosphorylation. The significance level was set at $P < 0.05$.

3.3. Results

3.3.1. Body mass, and muscle mass of NOS knockout mice

Body mass was not different between C57BL/6 control mice and eNOS$^{-/-}$ mice (28.1 ± 0.8 vs. 27.4 ± 1.3 g; P = 0.65; n = 6-8) or between nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ mice (24.1 ± 1.1 vs. 22.4 ± 0.6 g; P = 0.17, n = 6-7). EDL muscle mass was significantly lower in nNOSμ$^{-/-}$ compared with nNOSμ$^{+/+}$ mice (7.1 ± 0.2 vs. 8.6 ± 0.3 mg; P < 0.001; n = 12-14) consistent with our previous study (Hong *et al.*, 2015b), whereas EDL mass was similar between C57BL/6 control mice and eNOS$^{-/-}$ mice (10.5 ± 0.4 vs. 10.1 ± 0.4 mg; P = 0.49, n = 11-15).

3.3.2. Stretch-stimulated glucose uptake

To investigate the involvement of NOS in the regulation of glucose uptake in response to mechanical loading, we examined the effects on stretch-stimulated glucose uptake in EDL muscle of 1) pharmacological NOS inhibition, and 2) deletion of either eNOS or nNOSμ.

In muscles from C57BL/6 mice, passive stretch significantly increased glucose uptake approximately 2-fold compared with basal levels (P < 0.001) (Figure 1), which is in line with other stretch studies (Jensen *et al.*, 2014; Sylow *et al.*, 2015). Stretch significantly increase glucose uptake in muscle treated with either of the NOS inhibitors L-NMMA or L-NAME, and this was to a similar degree to control (stretch only) muscles (Figure 3.1).

In muscles from eNOS$^{-/-}$ mice, stretch increased glucose uptake approximately 2-fold from basal levels (P < 0.001) with a similar increase observed in C57BL/6 control mice (Figure 3.2A) Stretch also increased glucose uptake approximately 2-fold in muscles from nNOSμ$^{-/-}$

and nNOSμ$^{+/+}$ mice ($P < 0.001$). There was a genotype main effect indicating that muscles from nNOSμ$^{-/-}$ mice had elevated basal and stretch-activated glucose uptake compared with controls ($P = 0.02$) (Figure 3.2B). The delta stretch-stimulated glucose uptake (the difference between basal and stretch values) was similar between nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ mice (1.06 ± 0.22 and 1.04 ± 0.18 μmol/g/hr; $P > 0.05$). These results indicate that NOS is not necessary for normal increases in stretch-stimulated muscle glucose uptake.

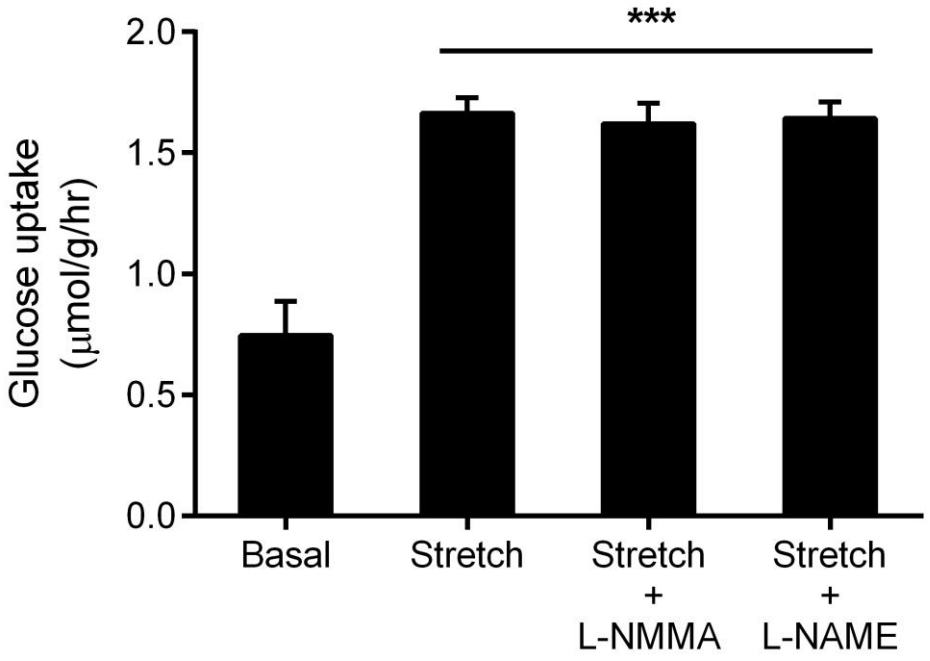

Figure 3.1 NOS inhibition does not attenuate stretch-stimulated glucose uptake. Stretch-stimulated 2-deoxyglucose uptake in EDL muscles from C57BL/6 mice incubated for 30 min with or without the NOS inhibitors L-NMMA (100 μM) or L-NAME (100 μM) (n = 4-10 per group). Data are means ± SEM. *** $P < 0.001$ vs. Basal.

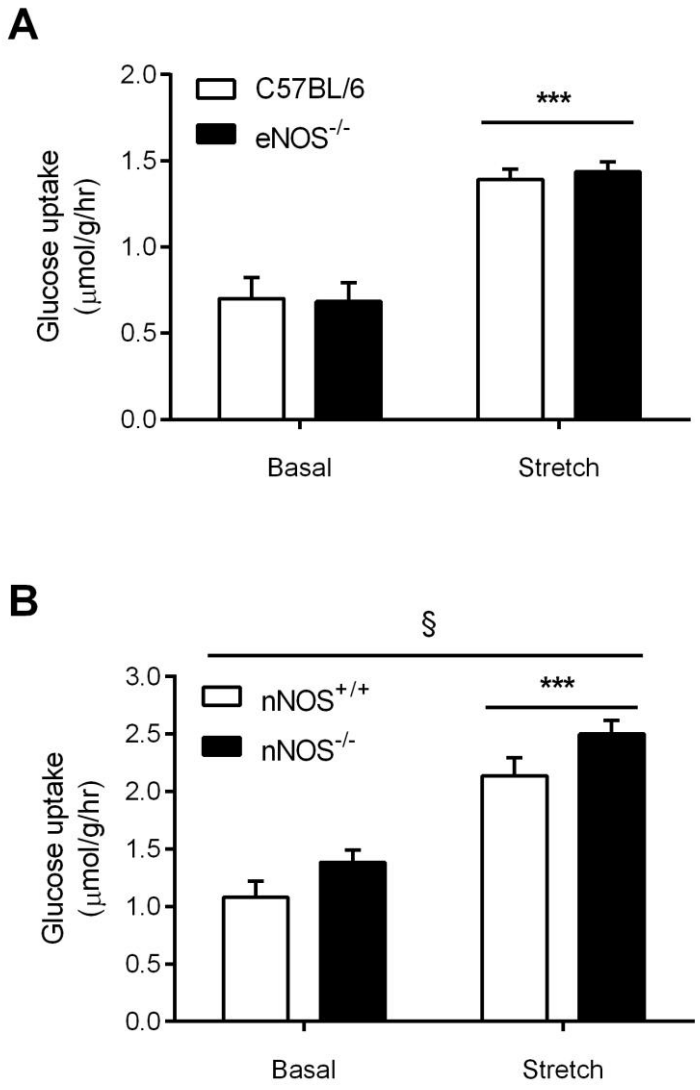

Figure 3.2 Deletion of eNOS or nNOSμ does not affect stretch-stimulated skeletal muscle glucose uptake. 2-deoxyglucose uptake at rest (basal) and during stretch in EDL muscles of A) C57BL/6 and $eNOS^{-/-}$ mice (n = 5-9 per group) and B) $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ mice (n = 6-7 per group). Data are means ± SEM. *** $P < 0.001$ compared to basal. **§** $P < 0.05$ main effect for genotype.

3.3.3. Effect of stretch on NOS activity and protein signalling

Stretch did not increase skeletal muscle NOS activity above basal levels (measure of contribution from both eNOS and nNOS) in EDL muscles from C57BL/6 mice (Figure 3.3A). This was consistent with the lack of effect of deletion of nNOSμ or eNOS, and the lack of effect of NOS inhibition on stretch-stimulated skeletal muscle glucose uptake.

To confirm that our stretch protocol was similar in activating signalling pathways previously reported in other *ex vivo* muscle stretching studies (Chambers *et al.*, 2009; Jensen *et al.*, 2014; Sylow *et al.*, 2015), we measured the phosphorylation status of p38 MAPK as well as the activation of the cytoskeletal regulator Rac1 (JeBailey *et al.*, 2004) by examining phosphorylation of the Rac1 downstream kinase PAK1/2 (Wang *et al.*, 2011; Sylow *et al.*, 2013b; Sylow *et al.*, 2015). Consistent with previous studies, stretch significantly increased the phosphorylation status of PAK1/2$^{Thr423/402}$ and p38 MAPK$^{Thr180/Tyr182}$ (~2-fold) ($P < 0.05$) (Figure 3.3 B and C) (Chambers *et al.*, 2009; Sylow *et al.*, 2013b; Jensen *et al.*, 2014; Sylow *et al.*, 2015). Skeletal muscle p-AMPK^{Thr172} did not increase with stretch which was also consistent with previous research (Chambers *et al.*, 2009; Jensen *et al.*, 2014; Sylow *et al.*, 2015) (Figure 3.3D). Likewise, stretch also failed to increase p-CaMKII^{Thr286} (Figure 3.3E).

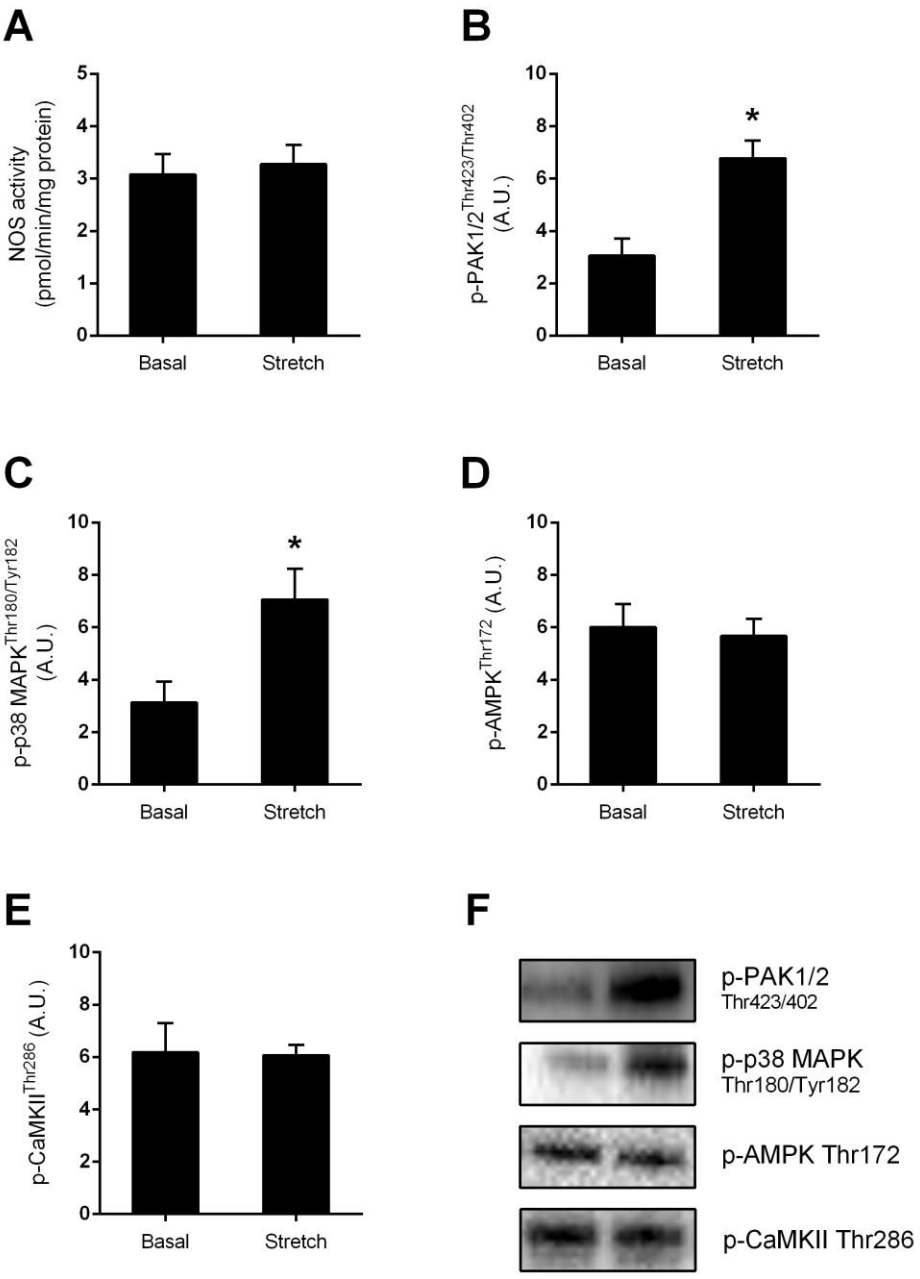

Figure 3.3 Passive stretch increases phosphorylation of skeletal muscle PAK1/2 and p38 MAPK independently of NOS activation. A) NOS activity of EDL muscles at rest (basal) or following passive stretch (n = 4 per group). Immunoblot quantifications for B) p-PAK1/2$^{Thr423/402}$, C) p-p38 MAPK$^{Thr180/Tyr182}$, D) p-AMPK^{Thr172}, E) p-CaMKII^{Thr286}, and E) representative immunoblots of EDL muscles at rest (basal) or following passive stretch (n = 4 per group). Data are means ± S.E.M.* $P < 0.05$ vs. Basal. A.U., Arbitrary unit.

3.4. Discussion

The major finding of this study was that skeletal muscle stretch-induced increases in glucose uptake are independent of NOS. Given that stretch activated Rac1 (as shown by increased PAK1/2 phosphorylation) but did not activate NOS, it appears that although nNOSμ is part of the dystrophin glycoprotein complex and linked to the cytoskeleton, stretch induces glucose uptake via the cytoskeleton independently of nNOSμ. In addition, eNOS is also not required for this process.

The lack of NOS activation in EDL muscles following stretch is in contrast with some studies whereby passive stretching was reported to increase measures of NO production (Tidball *et al.*, 1998; Tatsumi *et al.*, 2002; Zhang *et al.*, 2004; Wozniak & Anderson, 2009; Soltow *et al.*, 2010). However, it should be noted that most of these studies were conducted in cultured muscle cells where a much longer stretching/loading protocol (1 – 48 hours) was applied. Passive stretching applied to cells for prolonged periods has been shown to increase NOS protein content (Zhang *et al.*, 2004), and therefore the increased NO production reported in these chronic stretch studies may likely results from the increased NOS protein content rather than activation of the existing NOS. To our knowledge, only one previous study examined whether acute stretching was sufficient to stimulate NO production in mature intact muscle (Tidball *et al.*, 1998). Tidball and colleagues (1998) reported a significant increase (~20%) in NO production from isolated rat soleus muscles following a brief stretch. In that study, isolated muscles were stretched by 20% of their resting length for 2 minutes and NO production was determined by measuring the accumulation of nitrite in the incubation media from resting and stretched muscles. However, this method of NO measurement cannot define whether NOS is

the source of stretch-induced NO release since NOS and NOS inhibitors were not used to test this. Although NOS enzymes are considered the primary source of NO (Lau *et al.*, 2000), there is indeed evidence of NO generation independent of NOS in skeletal muscle (Lepore *et al.*, 1999). Furthermore, the relevance of NO in stretched soleus muscles from rats in regards to glucose uptake is unclear as it has previously been shown that NOS inhibition does not affect contraction-stimulated glucose uptake in rat soleus muscles (Higaki *et al.*, 2001) and our group has shown that NOS inhibition does not affect contraction-stimulated glucose uptake in mouse soleus muscle (Merry *et al.*, 2010c).

In the present study, NO production probably did not increase with stretch-stimulation given that NOS activity was not enhanced above resting levels. This inference is in agreement with a previous study where stretched single mature muscle fibres loaded with a NO-sensitive fluorescent probe (DAF-FM), which allowed for a more direct NO estimation, did not have an increase in NO production (Palomero *et al.*, 2012). That study is more in line with the stretching protocol of our present data in terms of stretching duration in that the single muscle fibres were stretched for an 8-minute period. The lack of stretch-induced NO production in mouse studies compared to evidence in rats indicates that mature rat and mouse muscle probably differ in their mechanical signalling properties. Indeed, the complexity of NO signalling is highlighted by indications that NOS activity during muscle activity is likely dependent on factors such as rodent type or strain, muscles examined, and muscle stretch/contraction protocol (discussed in Chapter Two).

NOS activity has been reported to increase immediately at the onset of stretching in a study where 2-second stretches were applied to muscle cells (Wozniak & Anderson, 2009). It

is possible that an initial burst of NOS activity/NO production with stretch diminishes rapidly over time and was therefore not detected at the time of muscle harvest in our study (15 min). Indeed, it has been shown that shear stress applied to endothelial cells resulted in a marked increase in NO production within 5 minutes followed by little additional NO production thereafter (Chang *et al.*, 2000). In another study, muscle NOS activity was significantly elevated 3 minutes following the induction of increased load applied to plantaris muscles in mice, and despite the continued load, NOS activity returned to baseline levels within 1 hour (Ito *et al.*, 2013). That study is difficult to interpret, however, given that tendons of synergist muscles were ablated resulting in "functional overload" of plantaris muscle and the time of overload was defined as 3 minutes after mice started walking post-surgery (Ito *et al.*, 2013). Nevertheless, the lack of increase in NOS activity with stretch fits with the observation that stretch-stimulated glucose uptake was not attenuated by NOS inhibitors or a lack of nNOSμ or eNOS.

The mechanism(s) by which NOS regulates contraction-stimulated glucose uptake remains to be determined. Since there is emerging evidence glucose uptake is largely regulated by distinct metabolic (AMPK)- and mechanical-dependent (Rac1) signalling arms during muscle contraction (Jensen *et al.*, 2014; Sylow *et al.*, 2017b), in this study we examined the potential involvement of NOS in a mechanical-dependent signalling pathway. The lack of involvement of NOS in stretch-stimulated glucose uptake and Rac1 activation indicates that NOS is not involved in the mechanical signalling arm, and by extension the possibility that NOS regulates glucose uptake during contraction via a mechanism coupled with metabolic disturbances. However, this would likely not involve AMPK (Sylow *et al.*, 2017b) given we

have previously shown that NOS appears to regulate muscle glucose uptake during contraction independently of AMPK (Merry *et al.*, 2010a; Merry *et al.*, 2010c). Nonetheless, it is important to note that in a recent study (Sylow *et al.*, 2017b), although contraction-stimulated glucose uptake was largely attenuated by blockade of both metabolic (AMPK) and mechanical (Rac1) signalling, some increase in glucose uptake with contraction was maintained, indicating other signalling pathways are likely at play. For example, mTORC2 signalling has been shown to be essential for muscle glucose uptake during exercise in mice independent of AMPK and Rac1 signalling (Kleinert *et al.*, 2017). Therefore, further work is required to examine the potential involvement of NOS in other signalling pathways during contraction.

The activation of Rac1 by contraction and stretch is associated with an increase in glucose transport in muscle (Sylow *et al.*, 2013b; Sylow *et al.*, 2015), however, the upstream signalling events involved are largely unknown. Rac1 contains a redox-sensitive motif and it has been reported that activation of Rac1 is favoured in the presence of reactive nitrogen species (Heo & Campbell, 2005). Exposure of C2C12 cells to a NO donor has previously been shown to induce the rapid activation of Rac1 and phosphorylation of its downstream kinase PAK1 (Godfrey & Schwarte, 2010), indicating that NO is sufficient to stimulate Rac1 activation. Conversely, nNOS and eNOS have been reported to be activated by Rac1 in human aortic endothelial cells (Selvakumar *et al.*, 2008). These results suggest that NO/NOS could be upstream and/or downstream of Rac1. We measured PAK1 phosphorylation as a surrogate for Rac1 activity and to investigate possible associations between NO and Rac1/PAK1 pathway. Our data indicate that NO is not necessary for Rac1 activation during stretching and vice versa, given that we observed an increase in stretch-stimulated phosphorylation of PAK1 (and

presumably Rac1 activity) without changes in NOS activation. Nonetheless, future work is required to clarify whether a NO-Rac1 interaction exists in skeletal muscle under situations where NO bioavailability is increased, such as during muscle contractions (Hong *et al.*, 2015b).

The role of p38 MAPK in regulating muscle glucose uptake during stretch is not clear as inhibition MAPK of p38 having been attenuate stretch-stimulated glucose uptake in one study (Chambers *et al.*, 2009), but not in another study (Jensen *et al.*, 2014). Nonetheless, the increased p38 phosphorylation with stretch despite lack of NOS activation suggests that similar to contractions (Merry *et al.*, 2010b), NOS and p38 are not connected in regulating glucose uptake during muscle activity.

Ca^{2+}/Calmodulin-dependent kinase II (CaMKII) has also been implicated in the regulation of muscle glucose uptake during contractions in mature muscle *in situ* (Witczak *et al.*, 2010), however, the inability of stretch to enhance the levels of phosphorylated $CaMKII^{Thr286}$ in our study suggest that CaMKII is not coupled with mechanical signalling mechanisms. This contrasts with a study where stretch-stimulated glucose uptake in C2C12 myotubes was blocked by a CaMK inhibitor (Iwata *et al.*, 2007). However, as discussed above, the pathways regulating stretch-stimulated glucose uptake potentially differ between *in vitro* and *ex vivo* models.

Although in this study NOS activity was not measured in the presence of L-NMMA or L-NAME, the dose of inhibitors used in this study were based on previously published work where the identical L-NMMA concentration was shown to block the increase in NOS activity during muscle contraction (Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015a) and where L-NAME has been shown to exert a similar dose-dependent effect on NOS activity (Roy

et al., 1998). Findings reported in Chapter Four of this thesis confirm that the concentration of L-NMMA was successful in blocking the increase in contraction-stimulated NOS activity. Furthermore, muscles lacking nNOSμ have been previously shown to exhibit only residual levels (~4%) of basal NOS activity with no detectable increase with muscle activity (Hong *et al.*, 2015a; Hong *et al.*, 2015b). As such, using both the inhibitor and knockout models which are sufficient in reducing NOS activity, we are confident in ruling out NOS as a required regulator of stretch-stimulated glucose uptake.

In conclusion, we have shown that passive stretching does not increase NOS activity in skeletal muscle and stretch-stimulated glucose uptake is not attenuated by either pharmacological inhibition of NOS or by deletion of eNOS or nNOSμ isoforms. Therefore, our results indicate that NOS signalling is not required for stretch-induced increases in skeletal muscle glucose uptake.

CHAPTER FOUR: THE ROLE OF NITRIC OXIDE SYNTHASE AND RAC1 IN REGULATING SKELETAL MUSCLE GLUCOSE UPTAKE DURING *EX VIVO* CONTRACTIONS

4.1. Introduction

Muscle contraction is a potent physiological stimulus that regulates GLUT-4 translocation and muscle glucose uptake via signalling pathways that are distinct to insulin-dependent signals (Richter & Hargreaves, 2013). Reduced insulin-stimulated muscle glucose uptake is a characteristic of type 2 diabetes, however, people with type 2 diabetes have normal muscle GLUT-4 translocation and glucose uptake during exercise (Martin *et al.*, 1995; Kennedy *et al.*, 1999; Kingwell *et al.*, 2002). This suggests that the signalling pathway(s) arising from the contracting muscles remains intact in people with type 2 diabetes (T2D) despite impairment to the insulin-signalling pathway. Therefore, defining the exact signal pathways involved in the contraction pathway has the potential to reveal therapeutic targets for the treatment of T2D.

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a small GTPase that is involved in the regulation of actin cytoskeletal dynamics (Ridley *et al.*, 1992). Initial cell culture studies demonstrate that Rac1 is involved in insulin-stimulated glucose uptake through coordination of actin filament dynamics (Khayat *et al.*, 2000; JeBailey *et al.*, 2004; JeBailey *et al.*, 2007).

More recently, a role for Rac1 as a key signalling protein involved in muscle glucose uptake during contraction or exercise has emerged. Indeed, electrical stimulation of muscle cells (Sylow *et al.*, 2016), muscle stretching (Sylow *et al.*, 2015), contractions and exercise in rodents (Sylow *et al.*, 2013b), and exercise in humans (Sylow *et al.*, 2013b) have been shown to activate Rac1 signalling as determined by either Rac1-GTP content or by phosphorylation of its downstream kinase PAK. Experimental models involving deletion of Rac1 or the presence of Rac1 inhibitors in mice have demonstrated that loss of Rac1 function attenuates muscle glucose uptake during *ex vivo* contractions or passive stretching (Sylow *et al.*, 2013b; Sylow *et al.*, 2015; Sylow *et al.*, 2017b) and during in vivo exercise (Sylow *et al.*, 2016; Sylow *et al.*, 2017b). Importantly, in Rac1-knockout (Rac1 KO) mice, muscle glucose uptake during treadmill running has been reported to be attenuated by approximately half, indicating that Rac1 is a major regulator of exercise-mediated glucose uptake (Sylow *et al.*, 2017b). The exact signalling pathway(s) by which Rac1 regulates muscle glucose uptake during contractions is not known. Studies have so far found that Rac1 signalling does not involve AMP-activated kinase (AMPK) (Sylow *et al.*, 2013b), mTORC2 (Kleinert *et al.*, 2017), TBC1D1/4 (Sylow *et al.*, 2017b) or p38 MAP-Kinase (Sylow *et al.*, 2017b). Therefore, interaction with other known candidates of muscle glucose uptake should be explored.

One such candidate is nitric oxide synthase (NOS) which has been postulated as a regulator of muscle glucose uptake (Richter & Hargreaves, 2013). Both muscle contraction and exercise increase skeletal muscle NOS activity (Roberts *et al.*, 1999; Merry *et al.*, 2010c; Linden *et al.*, 2011; Hong *et al.*, 2015b; Hong *et al.*, 2016) and there is evidence that NOS regulates muscle glucose uptake during contractions or exercise in both rodent studies (Balon

& Nadler, 1997; Roberts *et al.*, 1997; Stephens *et al.*, 2004; Ross *et al.*, 2007; Merry *et al.*, 2010b; Merry *et al.*, 2010c) and humans studies (Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Mortensen *et al.*, 2007). These studies have shown that the NOS inhibitors N^G -nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA) attenuate the normal increase in contraction- or exercise-stimulated NOS activity and glucose uptake. It should be noted, however, that these findings are controversial given that there are also several studies in both rodents (Etgen *et al.*, 1997; Higaki *et al.*, 2001; Rottman *et al.*, 2002; Hong *et al.*, 2015a) and humans (Heinonen *et al.*, 2013) that have failed to demonstrate an effect of NOS inhibition on skeletal muscle glucose uptake with contraction or exercise. Reasons for these discrepant results are not clear but possibly relate to differences in experimental design as have been discussed in detail within the literature review of this thesis. Nonetheless, the exact mechanisms by which NO regulates muscle glucose uptake during contraction/exercise are yet to be determined. Studies investigating molecular targets both upstream and downstream of NOS have thus far shown that NOS is probably not linked with cGMP (Merry *et al.*, 2010b), AMPK (Merry *et al.*, 2010c), TBC1D4 (Hong, 2014) or p38 (Merry *et al.*, 2010b).

Cell culture studies provide evidence of a potential Rac1-NOS signalling interaction. Reactive nitrogen species have been shown to directly interact with, and regulate, Rac1 activity by promoting nucleotide exchange *in vitro* (Heo & Campbell, 2005). In support, administration of a NO donor to C2C12 cells increases Rac1 activity (Godfrey & Schwarte, 2010). In another study, administration of bradykinin, to stimulate endogenous NO production in rabbit aortic cells, increased Rac1 activity, and this was blocked by the NOS inhibitor L-NAME (Eller-Borges *et al.*, 2015), suggesting that NO stimulated the increased Rac1 activity. These studies

provide rationale that Rac1 and NOS can interact, which prompted us to explore this in the context of skeletal muscle glucose uptake.

Therefore, in this study, we investigated whether a Rac1/NOS signalling nexus regulates muscle glucose uptake during *ex vivo* contraction of mouse extensor digitorum longus (EDL) muscle using Rac1 and NOS inhibition, and NOS KO mouse models. It was hypothesised that NOS is an upstream regulator of Rac1 in regard to the regulation of skeletal muscle glucose uptake during contraction.

4.2. Methods

4.2.1. Animals

Male C57BL/6JArc (referred herein to C57BL/6) mice (aged 12-16 weeks) (Animal Resources Centre (ARC), Perth, Australia) were used for inhibitor experiments. Male mice were used for NOS inhibitor experiments to be consistent with our previously published model (Merry *et al.*, 2010b; Merry *et al.*, 2010c). Male and female mice lacking nNOSμ ($nNOS^{-/-}$) and their wildtype littermates ($nNOS^{+/+}$) (aged 12-16 weeks) were used to examine the role of nNOSμ. We have previously used both male and female $nNOS^{-/-}$ mice and reported no sex differences in contraction-stimulated glucose uptake (Hong *et al.*, 2015b). $nNOS^{-/-}$ mice were originally purchased from Jackson Laboratories (Bar Harbor, ME; stock no. 002633) and backcrossed on a C57BL/6 background for at least 10 generations to establish an nNOS colony (as described in Chapter Three). The nNOS colony was maintained at The Florey Institute of Neuroscience and Mental Health, Parkville, Australia and mice were transported to Victoria University where they were housed for at least one week prior to experimentation. Female mice lacking eNOS ($eNOS^{-/-}$) (Monash Animal Services, Melbourne, Australia) and female age-matched C57BL/6 mice (ARC, Perth, Australia) (aged 12-16 weeks) were used for experiments examining the role eNOS. As described in Chapter Three, wildtype littermate mice from the eNOS mouse colony were not available and therefore we used age- and sex-matched C57BL/6 as controls since the eNOS colony had been generated on this background. The use of female mice only for eNOS experiments was due to limited availability of male mice. All animal experimentation was conducted at the Institute of Sport, Exercise & Active Living (ISEAL), Victoria University, Melbourne with the prior approval from Victoria University Animal Ethics

Committee Melbourne and adhered to the Australian Code of Practice for the use and care of animals for scientific purposes as described by the National Health and Medical Research Council (NHMRC) of Australia. Mice were housed in standard cages and maintained at ~21°C on a 12 hour dark/light cycle with access to water and standard rodent chow *ad libitum.*

4.2.2. Experimental Procedures

Extensor digitorum longus (EDL) muscles were excised from anaesthetised mice (sodium pentobarbitone 70 mg/kg IP) and suspended in organ baths (MultiMyograph System, Danish Myotechnology, Aarhus, Denmark). The rationale for the use of EDL muscles has been described in Study 1 of this thesis (see 3.2 Methods). Muscles were equilibrated for 60 min in Krebs-Ringer-Henseleit buffer (KHB) consisting of (mM): NaCl 118.5, NaHCO3 24.7, KCl 4.74, MgSO4 1.18, KH2PO4 1.18, CaCl2 2.5, (pH 7.4) supplemented with 0.01% BSA, 8 mM mannitol and 2 mM sodium pyruvate. Incubation media was maintained at 30°C and continuously oxygenated with gas containing 95% O_2 and 5% CO_2. Muscles were suspended at optimal length determined by incrementally adjusting the resting length of the muscle to which elicited the greatest twitch force. Muscles were then stimulated to contract for 10 minutes or remained at rest. Because there is indication that NOS is activated in an intensity-dependent manner (Silveira et al., 2003; Lee-Young et al., 2009), the magnitude by which NOS may regulate contraction-stimulated glucose uptake could also be related to the contraction intensity. Therefore, in this study we examined two stimulation protocols. Stimulation protocol one (presented in Figure 4.2-4, 4.6 and 4.7) comprised of square-wave 0.2 ms pulses, 60 Hz, 12 volts, 350 ms train duration, at 12 contractions per minute. This protocol has previously been used by our group and showed that NOS inhibition attenuates the increase in contraction-

stimulated glucose uptake in isolated mouse EDL muscle (Merry *et al.*, 2010c; Hong *et al.*, 2015b). Stimulation protocol 2 (presented in Figure 4.5) consisted of square-wave 0.2 ms pulses, 100 Hz, 30 volts, 2000 ms train duration at 4 contractions per minute (Sylow *et al.*, 2013b). Pilot testing of these protocols showed that protocol two elicited a greater decline in force production over the first five minutes of the stimulation procedure (Appendix B). For inhibitor experiments, muscles were either incubated in the presence of N^G-monomethyl-L-arginine (L-NMMA, 100 μM) for 30 min (Higaki *et al.*, 2001; Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b), or Rac1 inhibitor II (15 μM) for 60 min (Sylow *et al.*, 2015; Sylow *et al.*, 2017b), or no treatment (control). This Rac1 inhibitor has been reported to target Rac1 without targeting other RhoGTPases Cdc42 or RhoA (Ferri *et al.*, 2009). Inhibitors were dissolved in water as previously conducted for published L-NMMA experiments (Hong *et al.*, 2015b). Inhibitors were also present during the glucose uptake measurement.

For NO donor experiments, muscles were pre-incubated in KHB for 60 min, with the NO donor Diethylenetriamine NONOate (DETA/NO) (500 μM) present for the final 30 min (Merry *et al.*, 2010b), or DETA/NO combined with Rac1 inhibitor II (15 μM) (Sylow *et al.*, 2013b), or received no treatment (basal). DETA/NO was prepared immediately prior to use and dissolved in water. At the concentration used DETA/NO has been shown to significantly increase resting glucose uptake in isolated EDL muscles (Merry *et al.*, 2010b). Where the Rac1 inhibitor was used, it was present for the entire 60min pre-incubation period (Sylow *et al.*, 2013b). Inhibitors were also present when skeletal muscle glucose uptake was being measured.

4.2.3. Muscle Processing

To generate lysates for immunoblotting and NOS activity measurement, whole frozen EDL muscles were homogenised in ice-cold buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 5 µl/ml protease inhibitor mixture, 50 mM sodium fluoride, and 5 mM sodium pyrophosphate] by steel bead for 2 x 30 s 30 Hz (TissueLyser, Qiagen, Valencia, CA), followed by end-over-end rotation for 30 min at 4°C. The lysate was centrifuged at 10,000 g for 20 min at 4°C, and the supernatant collected for NOS activity measurement performed as described below. For immunoblotting, an aliquot was collected prior to the centrifugation step and diluted in sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT and 0.01% bromophenol blue) (Hong *et al.*, 2015b). These samples were boiled for 10 min at 95°C before being subjected to SDS-PAGE. Protein concentration was determined by the Red660 protein assay kit (G Biosciences, St Louis, MO).

4.2.4. Immunoblotting

Total protein (5 µg) was separated by SDS-PAGE using stain-free gels (Bio-Rad, Hercules, CA) and semi-dry transferred (TransBlot Turbo system, Bio-Rad, Hercules, CA) to PVDF membranes. Membranes were then blocked for 1 hour at room temperature (5% skim milk in TBST), before being probed with p-PAK1/2$^{Thr423/Thr402}$ antibody (1:500) (Cell Signalling Technology, Beverly, MA) and p-AMPK antibody (1:1000) overnight at 4°C. The following day, membranes were incubated with HRP-secondary antibody for 1 hour at room temperature. Protein bands were visualised using Bio-Rad ChemiDoc imaging system and

enhanced chemiluminesence substrate (SuperSignal West Femto, Pierce, MA), and quantified using ImageLab software (Bio-Rad). Stain free images of the gels used were collected to quantify protein loading and analysis of phosphorylated proteins are reported as normalised to stain free quantification.

4.2.5. NOS activity and Glucose Uptake Measurement

NOS activity was determined on muscle lysates by measuring the conversion of radiolabelled L-[^{14}C] arginine to L-[^{14}C] citrulline (Lee-Young *et al.*, 2009; Hong *et al.*, 2015b) as described in Chapter Three, muscle glucose uptake was measured during the final 5 min of contractions for contraction protocol one, or the final 10 min of NO donor experiments by exchanging the incubation buffer with buffer containing 1 mM 2-deoxy-D-[1,2-^{3}H] glucose (0.128 µCi/mL) and 8 mM D-[^{14}C] mannitol (0.083 µCi/mL) (Perkin Elmer, Boston, MA) (Merry *et al.*, 2010b; Hong *et al.*, 2015b). For contraction protocol two, glucose uptake was measured over the entire 10 min contraction period as this followed a previously established protocol (Jensen *et al.*, 2007b; Sylow *et al.*, 2013b). Determination of glucose uptake was performed as described in Chapter Three.

4.2.6. Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0 software. Glucose uptake, protein phosphorylation status and NOS activity were analysed using one (treatment)- and two (treatment and genotype)-factor ANOVA where appropriate. Fisher's least significance test was performed if the ANOVA revealed a significant

difference. Student’s t-test was used to compare morphological characteristics between each genotype and its relevant control. The significance level was set at $P < 0.05$.

4.3. Results

4.3.1. NO donor-stimulated glucose uptake is blocked by Rac1 inhibition

To investigate whether NO is sufficient to mediate glucose uptake via a mechanism involving Rac1, we incubated EDL muscles with or without the NO donor, DETA/NO (500 µM), or with DETA/NO combined with Rac1 inhibitor II. DETA/NO significantly increased resting glucose uptake approximately 1.5-fold which is consistent with our previous findings (Merry *et al.*, 2010b). This increase in glucose uptake was completely abolished in the presence of Rac1 inhibitor II (Figure 4.1).

4.3.2. NOS activity, Rac1/PAK1 and AMPK signalling during contraction

Contractions significantly increased NOS activity approximately 3-fold above resting levels and L-NMMA completely blocked this increase demonstrating the efficacy of L-NMMA at the dose used (100 µM) (Figure 4.2A). This is in agreement with our previous studies in C57BL/6 mice (Merry *et al.*, 2010b), AMPK dominant negative mice (Merry *et al.*, 2010c), and wildtype littermate controls of nNOS KO mice (Hong *et al.*, 2015b). During contractions in the presence of the Rac1 inhibitor, NOS activity increased to almost a similar degree as contractions alone (~2.5-fold), however, this was not statistically significant (P = 0.06) (Figure 4.2A).

Phosphorylation status of the Rac1 downstream target PAK (PAK1/2 $^{\text{Thr } 423/402}$) was used as a surrogate marker for Rac1 activation (Sylow *et al.*, 2013b). Contractions significantly increased p-PAK1/2 approximately 2-fold above resting levels and this was significantly

attenuated by the Rac1 inhibitor. Contraction significantly increased p-PAK1/2 in L-NMMA-treated muscles indicating that NOS activity is not an upstream regulator of Rac1/PAK signalling (Figure 4.2B).

Because the phosphorylation status of AMPKα Thr172 was measured in our previous NOS inhibitor studies (Merry *et al.*, 2010b; Merry *et al.*, 2010c), we used this to provide an indication that the contraction protocol used in this study elicited a similar degree of metabolic stress to that of our previous studies. Contractions significantly increased p-AMPKα Thr172 by approximately 4-fold and as expected, this was not altered by either L-NMMA or Rac1 inhibitor (Figure 4.2C).

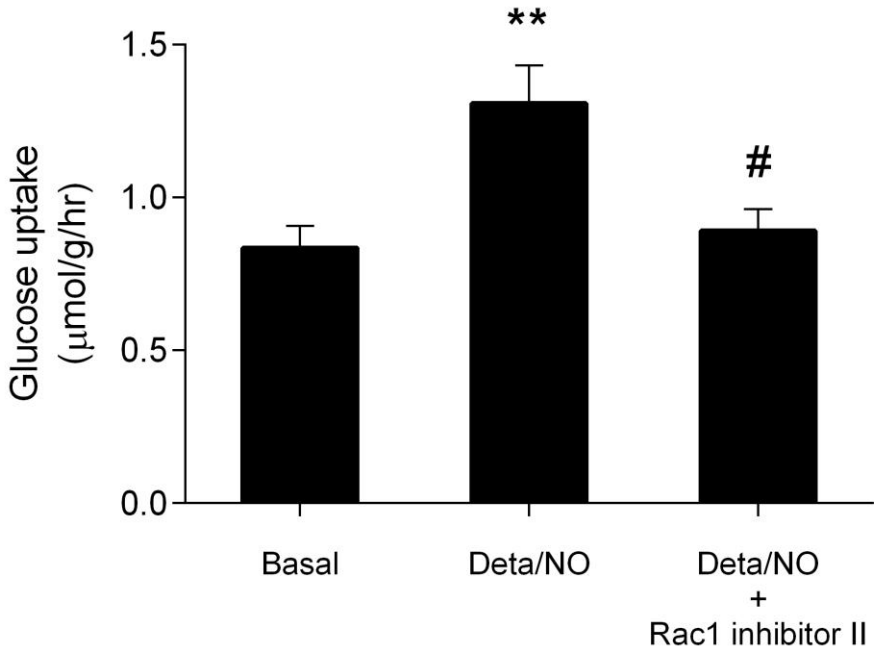

Figure 4.1 NO donor-stimulated glucose uptake is blocked by Rac1 inhibition. Bar graph showing 2-deoxyglucose uptake in EDL muscles from C57BL/6 mice at rest (basal) or in the presence of the NO donor DETA/NO (500 µM) or combined incubation with DETA/NO and Rac1 inhibitor II (15 µM) (n = 9 basal, 16 DETA/NO, 9 DETA/NO + Rac1 inhibitor II). Data are means ± SEM. ** $P < 0.01$ compared to basal. # $P < 0.05$ compared to DETA/NO.

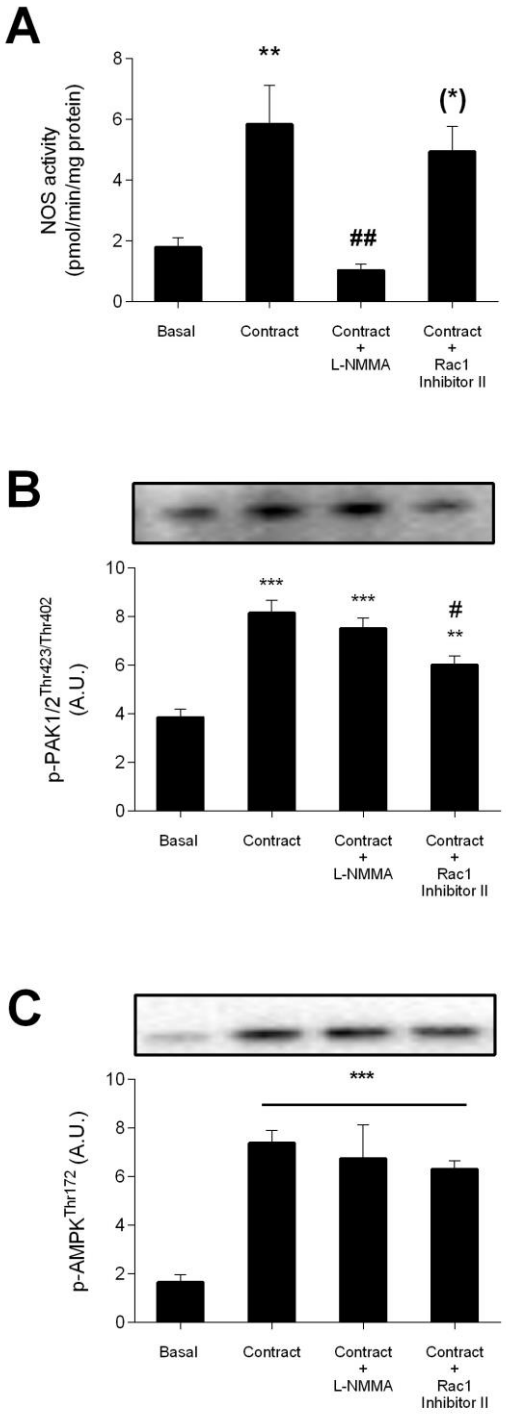

Figure 4.2 (Continued on the following page)

Figure 4.2 Contraction-stimulated NOS activity, p-PAK1/2, and p-AMPK. Bar graph showing effect of muscle contractions EDL muscles from C57BL/6 mice with or without L-NMMA (100 μM) or Rac1 inhibitor (15 μM) on A) NOS activity, B) p-PAK1/2 and representative blot, and C) p-AMPK and representative blot (n = 8 basal, 11 contract, 4 contract + L-NMMA, 5 contract + Rac1 inhibitor). Data are means ± SEM. **/*** P < 0.01/0.001. #/## P < 0.05/0.01 compared to contract only. (*) P = 0.06 compared to basal. A.U., arbitrary units.

4.3.3. Contraction stimulated glucose uptake is regulated by Rac1 but not NOS

Contraction significantly increased skeletal muscle glucose uptake approximately 1.8-fold above resting levels (Figure 4.3). The Rac1 inhibitor significantly attenuated the increase in contraction-stimulated glucose uptake by approximately 40%. In the presence of L-NMMA, contraction-stimulated glucose uptake was similar to control muscles with 1.8-fold increase in glucose uptake (Figure 4.3).

4.3.4. Muscle contraction forces with NOS and Rac1 inhibition

The decline in force production over the 10 min stimulation period was similar in L-NMMA and Rac1 treated muscles to that of controls (untreated) (Figure 4.4A). Likewise, initial peak contraction force was similar in L-NMMA and Rac1 treated muscles to that of controls (untreated) (Figure 4.4B), indicating that the inhibitors did not affect force output.

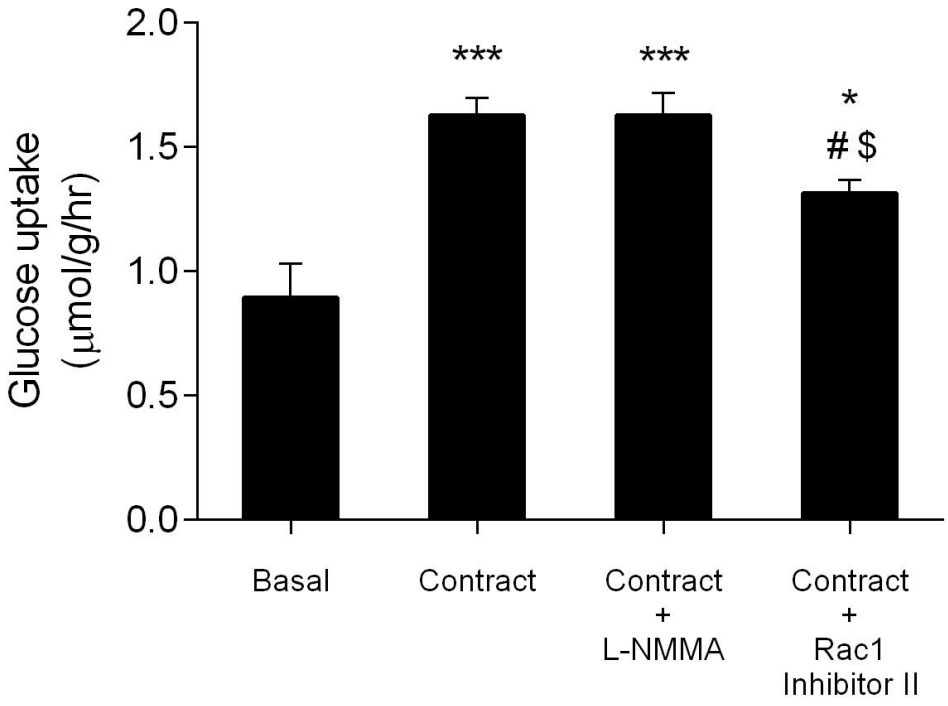

Figure 4.3 Contraction-stimulated glucose uptake in the presence of NOS or Rac1 inhibitors. Bar graph showing contraction-stimulated 2-deoxyglucose uptake in EDL muscles from C57BL/6 mice incubated with or without L-NMMA (100 µM) or Rac1 inhibitor II (15 µM) (n = 4 basal, 14 contract, 10 contract + L-NMMA, 4 Contract + Rac1 inhibitor). Data are means ± SEM */*** $P < 0.01/0.001$ compared to Basal. # $P < 0.05$ compared to Contract. $ $P < 0.05$ compared to Contract + L-NMMA.

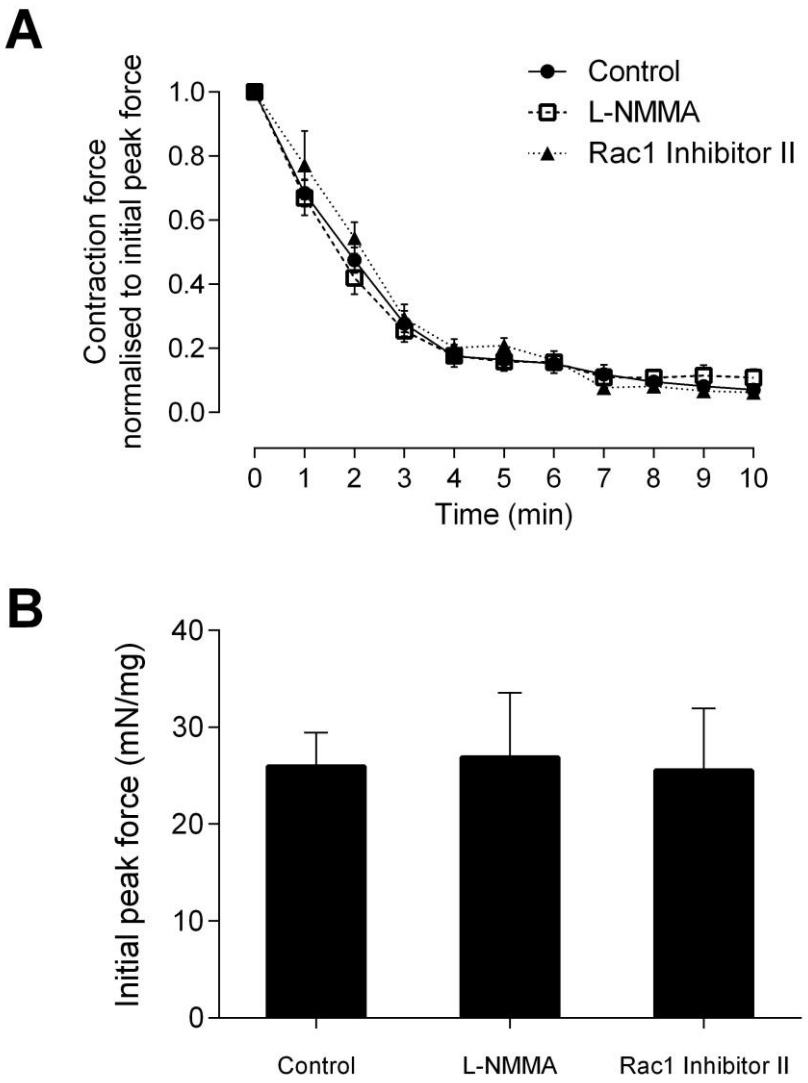

Figure 4.4 Muscle contraction force in the presence of NOS or Rac1 inhibitors. A) EDL muscle contraction force profile during the 10 min electrical stimulation period. Force is normalised to initial peak contraction force. B) Bar graph showing initial peak contraction force (n = 14 control, 10 L-NMMA, and 4 Rac1 inhibitor II). Muscles were treated with either L-NMMA (100 µM), Rac1 inhibitor II (15 µM), or remained untreated (control). Data are means ± SEM.

4.3.5. L-NMMA does not attenuate glucose uptake during higher intensity contractions

Because we failed to observe an effect of L-NMMA on glucose uptake as reported using the identical stimulation protocol to previous studies from our lab (Merry *et al.*, 2010c; Hong *et al.*, 2015b), we also examined the potential effect of L-NMMA on glucose uptake during contractions using a higher-intensity stimulation protocol. Pilot work showed that this stimulation protocol resulted in a more rapid decline in contraction force during the first 5 minutes of stimulation (Appendix A). The decline in force production over the 10 min stimulation period was similar between L-NMMA and control (untreated) muscles (Figure 4.5A). Likewise, the initial peak contraction force was similar between L-NMMA and control (untreated) muscles (Figure 4.5B). Using this protocol, as expected, contractions significantly increased skeletal muscle glucose uptake approximately 2-fold above resting levels. In accord with our initial contraction and NOS inhibitor data (Figure 4.3), contraction-stimulated glucose uptake using this higher intensity protocol was similar between muscles treated with or without L-NMMA (~2-fold increase) ($P = 0.29$, Contraction vs Contraction + L-NMMA) (Figure 4.5C).

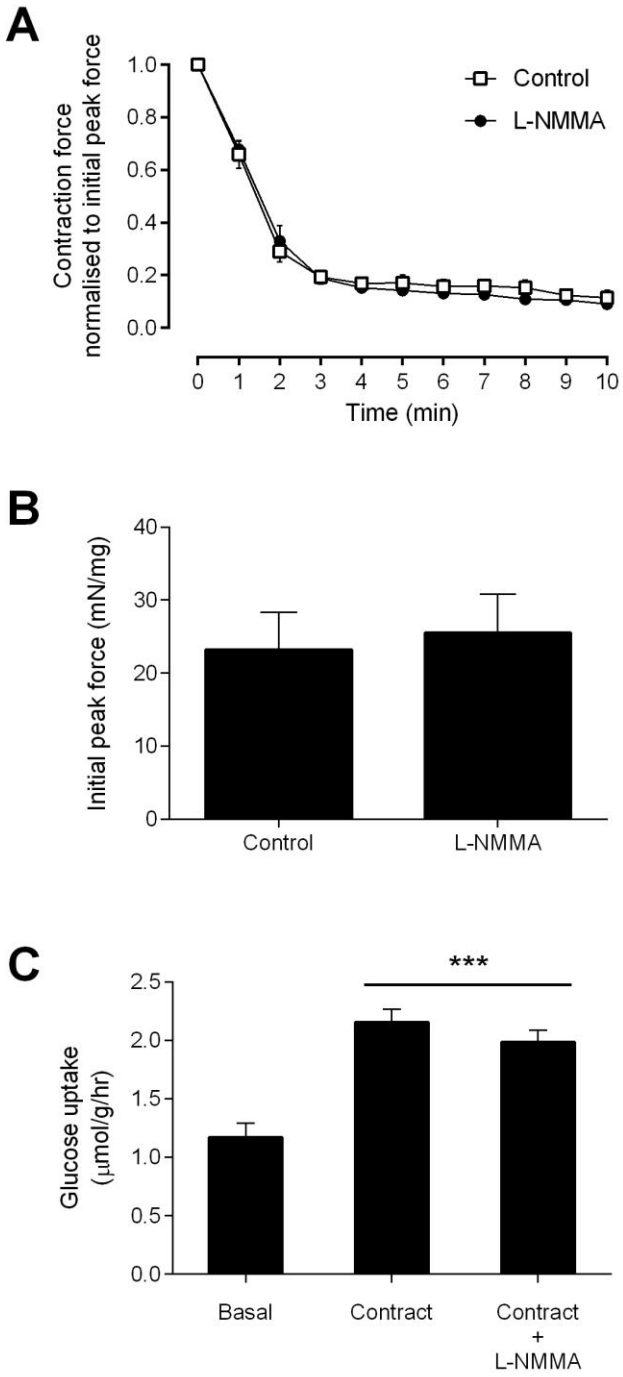

Figure 4.5 NOS inhibition does not attenuate glucose uptake during contraction at a higher intensity. A) Force development during electrically-stimulated contractions normalised to initial peak and B) Initial peak contraction force (n = 6 control, 7 L-NMMA). C) 2-deoxyglucose uptake at rest (basal) or during contractions with or without L-NMMA (100 µM). Electrical stimulations were elicited at 100 Hz, 30 volts, 2 s contraction trains every 15 s (n = 6 Basal, 6 Contract, 7 Contract + L-NMMA -11 per group). Data are means ± SEM. *** P < 0.001 compared to basal.

4.3.6. Deletion of eNOS and nNOSμ does not attenuate contraction-stimulated glucose uptake

To further examine the role of NOS/NO in muscle glucose uptake, we next conducted experiments using eNOS KO and nNOSμ KO mice. Contractions increased glucose uptake in muscles from $eNOS^{-/-}$ mice by approximately 2-fold and this was similar to muscles from C57BL/6 control mice (Figure 4.6A). Likewise, contractions elicited a 2-fold increase in glucose uptake in both $nNOS^{-/-}$ and $nNOS^{+/+}$ mice (Figure 4.6B) which is consistent with our previous study using these mice (Hong *et al.*, 2015b). There was tendency for a main of effect of genotype ($P = 0.08$).

The decline in force production over the 10 min stimulation period was similar between $eNOS^{-/-}$ and C57BL/6 control mice and likewise, similar between $nNOS^{-/-}$ and $nNOS^{+/+}$ mice. Initial peak contraction force was similar between $eNOS^{-/-}$ and C57BL/6 control mice. The control mice used for the eNOS experiments (C57BL/6) were of similar body mass and EDL muscle mass to that of $eNOS^{-/-}$ mice. $nNOS^{-/-}$ mice tended to have a lower body mass ($P = 0.06$) compared to $nNOS^{+/+}$ mice and EDL muscle mass was significantly lower in $nNOS^{-/-}$ mice compared with $nNOS^{+/+}$ mice (Figure 4.7).

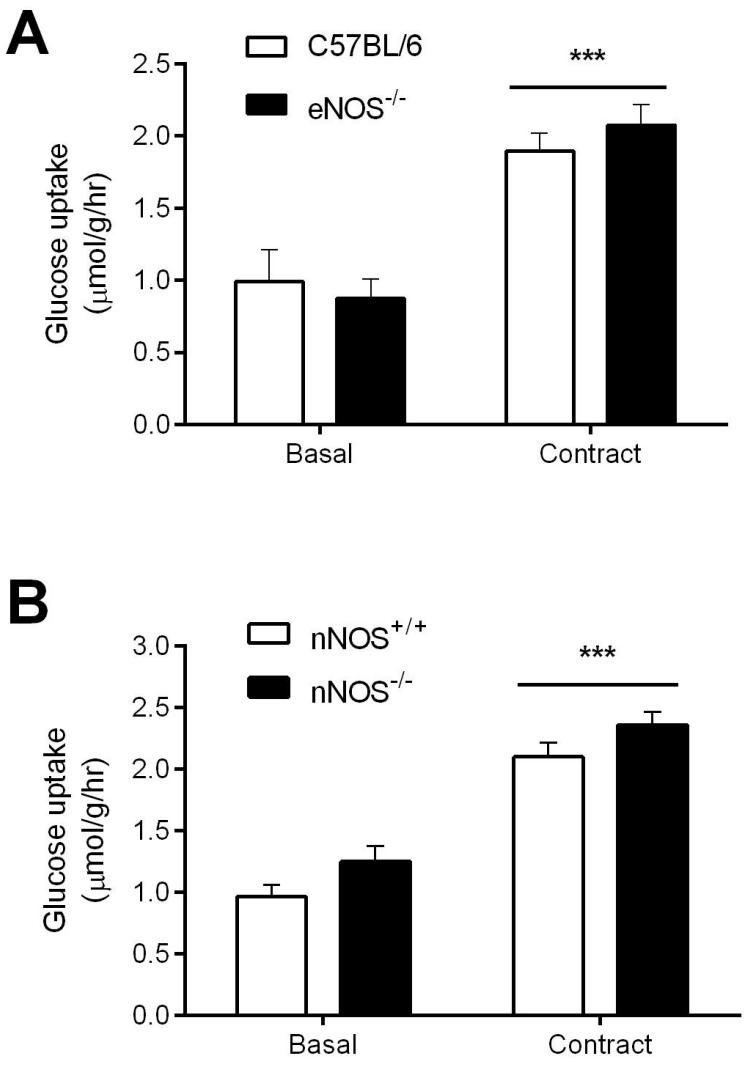

Figure 4.6 Loss of eNOS or nNOS does not affect contraction-stimulated glucose uptake. Bar graph showing 2-deoxyglucose uptake at rest (basal) and during contractions in EDL muscles from A) C57BL/6 (n = 3 Basal, 7 Contract) and $eNOS^{-/-}$ (n = 2 Basal, 6 Contract) mice and B) $nNOS^{+/+}$ (n = 3 Basal, 12 Contract) and $nNOS^{-/-}$ (n = 4 Basal, 10 Contract) mice. Data are means ± SEM. *** $P < 0.001$ compared to basal.

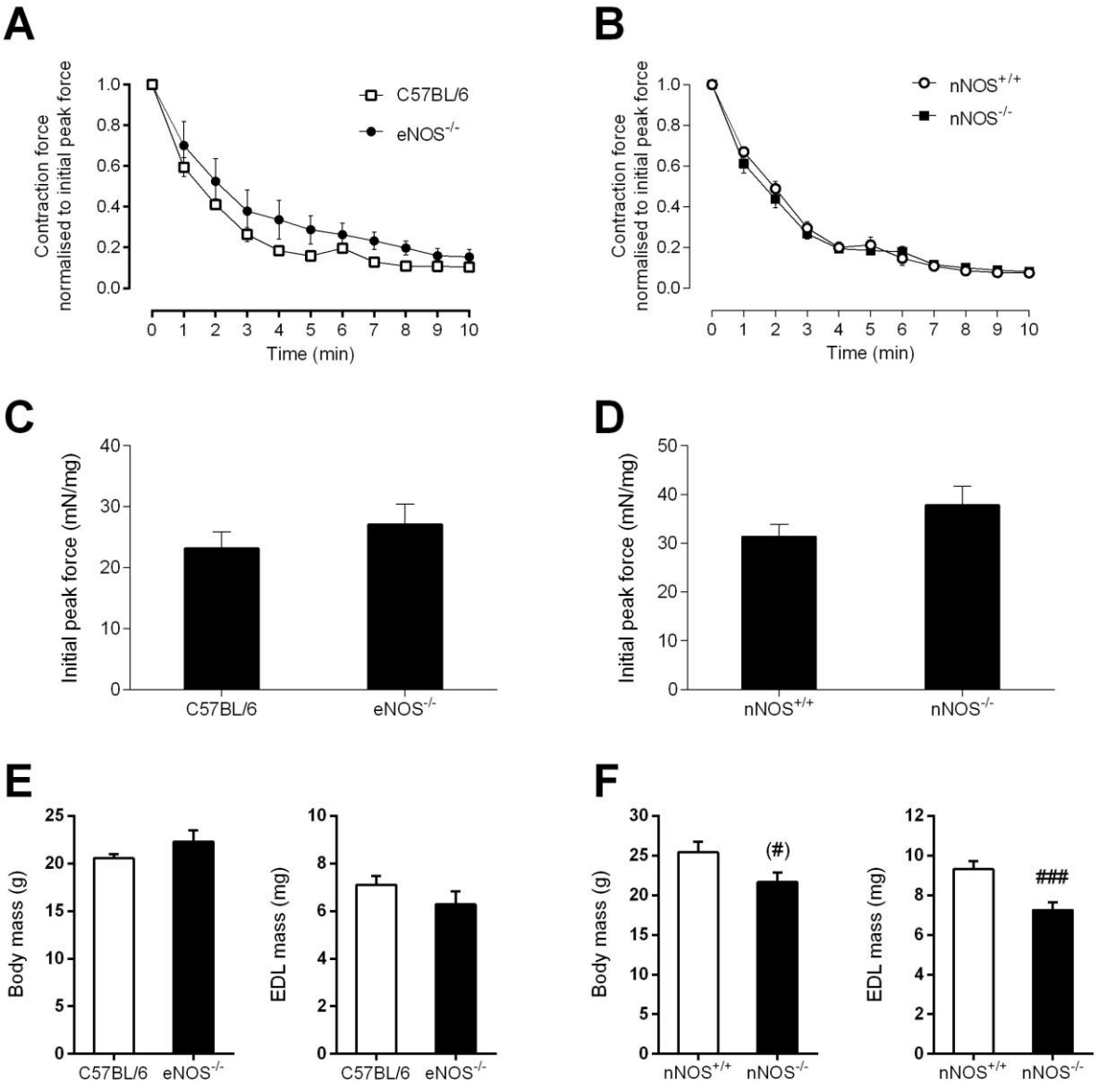

Figure 4.7 (Continued on the following page)

Figure 4.7 eNOS and nNOS KO contraction forces, body mass and muscle mass. Force development during 10 min electrical stimulations in EDL muscles from A) C57BL/6 and $eNOS^{-/-}$ mice (n = 6-7 per group) and B) $nNOS^{+/+}$ and $nNOS^{-/-}$ mice (n = 10-12 per group). Muscle force presented as normalised to initial peak contraction force. Bar graphs showing initial peak contraction from C) C57BL/6 and $eNOS^{-/-}$ mice (n = 6-7 per group) and D) $nNOS^{+/+}$ and $nNOS^{-/-}$ mice (n = 10-12 per group). Bar graphs showing body mass and EDL mass from E) C57BL/6 and $eNOS^{-/-}$ mice (n = 8 mice for C57BL/6 and 4 mice for $eNOS^{-/-}$) and F) $nNOS^{+/+}$ and $nNOS^{-/-}$ mice (n = 11 mice for $nNOS^{+/+}$ and 8 mice for $nNOS^{-/-}$). Data are means ± SEM. *** $P < 0.001$ compared to basal. (#) $P < 0.1$, ### $P < 0.001$ compared to $nNOS^{+/+}$.

4.4. Discussion

In the present study, we tested the hypothesis that nitric oxide synthase (NOS) and Rac1 interact to regulate muscle glucose uptake during contractions. Increasing NO concentration in isolated mouse EDL muscles by the administration of a NO donor increased resting muscle glucose uptake, and this increase was blocked in the presence of Rac1 inhibitor indicating that Rac1 may serve as a downstream target in NO-mediated glucose uptake. Rac1 inhibition attenuated the normal increase in contraction-stimulated glucose uptake. Surprisingly, however, when attempting to further examine this signalling pathway during muscle contractions, we found that contraction-stimulated glucose uptake was normal despite inhibition of NOS activity by NOS inhibitors or a lack of nNOS or eNOS. These findings were in contrast to previous findings from our group (Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b), despite using a similar experimental design, and challenge the hypothesis that NO is an important regulator of muscle glucose uptake during contractions.

An initial aim of this study was to further investigate Rac1 signalling by probing a potential interaction with NOS. In this study, we measured phosphorylation of the Rac1 downstream kinase PAK1/2 as a surrogate for Rac1 activity. Consistent with previous studies (Sylow *et al.*, 2013b; Sylow *et al.*, 2017b), we found that Rac1 inhibition attenuated the contraction-stimulated increase in p-PAK1/2 (Figure 4.2) and attenuated glucose uptake during contraction by approximately 40% (Figure 4.3). The observation that NOS activity

was increased with contractions in the presence of Rac1 inhibitor suggests that activation of Rac1 is not required to increase NOS activity. In support, dissociation between Rac1 and NOS was indicated in Chapter Three of this thesis, where it is shown that passive stretching increases PAK phosphorylation and presumably Rac1 activity despite a lack of increase in NOS activity above resting levels. In the present study, we also examined whether NOS can act as an upstream regulator of Rac1-mediated glucose uptake. Incubation of muscles with the NO donor, DETA/NO significantly increased glucose uptake in resting EDL muscles, consistent with our previous observation (Merry *et al.*, 2010b). When muscles were co-incubated with the Rac1 inhibitor, the NO-donor mediated glucose uptake was completely blocked, suggesting that NO may be sufficient to regulate glucose uptake via Rac1 (Figure 4.1). Unfortunately, we did not collect sufficient tissue to perform immunoblotting to confirm whether p-PAK1 was indeed increased with the NO donor. However, in support of our data, it has been previously reported that incubation of muscle cells in culture with a NO donor resulted in an increase Rac1 activity and phosphorylation of the downstream kinase PAK1 (Godfrey & Schwarte, 2010). That the Rac1 inhibitor was able to block the increased glucose uptake in response to the NO-donor in the current study indicates that the increased level of NO likely activated Rac1/PAK signalling. Nonetheless, the physiological relevance of this observation is uncertain given we also show that blocking NOS activity during muscle contractions did not affect the increase in skeletal muscle glucose uptake. Exogenous NO can result in different signalling mechanisms to that of endogenously produced NO (Iwakiri *et al.*, 2006). Indeed, we have previously shown that inhibitors of cGMP/PKG signalling block

basal (non-contraction) glucose uptake in response to exogenous NO, but not in response to increase in NOS activation during contraction (Merry *et al.*, 2010b), suggesting that the mechanisms by which NO donors stimulate basal glucose uptake is not indicative of contracting conditions.

In this study, although the increase in p-PAK1/2 with contraction was not fully blocked by the Rac1 inhibitor, the partial reduction in contraction-stimulated glucose uptake (~40%) is in line with previous studies using the same Rac1 inhibitor concentration (15 µM) (Sylow *et al.*, 2013b; Sylow *et al.*, 2017b). Studies using Rac1 knockout mice, which lack an increase in Rac1/PAK signalling with contraction, have reported that contraction-stimulated glucose uptake was attenuated by ~30-40% (Sylow *et al.*, 2013b; Sylow *et al.*, 2017b). It is likely that several proteins are involved in parallel in the regulation of muscle glucose uptake during contraction (Richter & Hargreaves, 2013), and as such, blocking one protein results in only partial attenuation of glucose uptake due to the presence of other regulators that likely still are at play. In our study, the presence of a Rac1 inhibitor did not fully block the increase in p-PAK1/2, however, the reduction in glucose uptake by the inhibitor was similar to that of published Rac1 KO studies (~40%). As such, it is likely we maximally prevented the degree to which Rac1 signalling contributes to muscle glucose uptake during contraction.

In our previously published studies, NOS inhibition was found to attenuate the normal increase in skeletal muscle glucose uptake during contraction by ~40% (Merry *et al.*, 2010c) and~50% (Merry *et al.*, 2010b) in initial studies, and more recently by ~25% (Hong *et al.*,

2015b). Therefore, it was surprising to find in the present study that our experimental model failed to find an effect of NOS inhibition on skeletal muscle glucose uptake with contraction (Figure 4.3). In the present study significant increases in skeletal muscle NOS activity with contraction, and blockade of this increase with L-NMMA indicates our contraction protocol was sufficient to probe the involvement of NOS and suggests that NOS is not a required mediator of muscle glucose uptake.

Nonetheless, given the notion that NOS plays a role in glucose uptake at higher contraction intensities, we also investigated the effect of L-NMMA using a second stimulation protocol which resulted in a more rapid decline in muscle force. Consistent with our initial finding, we again failed to observe an effect of L-NMMA on contraction-stimulated glucose uptake using this stimulation protocol (Figure 4.5). In support of the present findings, other preliminary experimental work from our laboratory also found that contraction-stimulated glucose uptake was similar between L-NMMA treated and control muscles (S. Betteridge, M. Zhang, AC Betik, GK McConell, *unpublished observations*). Furthermore, we are aware of preliminary data from Prof Erik Richter's group in Denmark who also report that L-NMMA (100 μM) does not attenuate glucose uptake in contracting EDL muscles from C57BL/6 mice (L. Sylow, University of Copenhagen, *personal communication*).

Previous rodent studies examining the effect of NOS inhibition on skeletal muscle glucose uptake during contraction have resulted in conflicting findings. However, drawing

conclusions from these studies is difficult given a number of experimental variations exist such as differences in rodent species/strains, muscles examined, inhibitors used, timing of glucose uptake measurements compared with inhibition, exercise/contraction model (*in vivo*, *in situ*, *ex vivo*), and exercise/muscle stimulation intensity (see section 2.3.8 of this thesis and (McConell *et al.*, 2012)). NOS appears to be activated in an intensity-dependent manner. Indeed, electrical stimulation of muscle cells in culture increased NO production at higher but not lower stimulation intensities (Silveira *et al.*, 2003). In support, it has previously been shown in mice treadmill running at 45% maximum speed failed to increase muscle NOS activity while running at 70% maximum speed resulted in a significant increase in NOS activation (Lee-Young *et al.*, 2009). In accord, NOS inhibition has been shown to attenuate the increase in muscle glucose uptake during *in situ* contractions in rats at higher but not lower stimulation intensities (Inyard *et al.*, 2007). There are also indications from human studies using single-leg exercising models that NOS inhibitors appear to affect glucose uptake during moderate-intensity exercise (Mortensen *et al.*, 2007) but not at a lower exercise intensity (Heinonen *et al.*, 2013). It is important to note that a number of previously published studies which report no effect of NOS inhibitors on glucose uptake (see section 2.3.8) did not include measures of NO or NOS activation with contraction/exercise, or did not utilise experimental protocols in which were known to activate NOS. Such studies should be interpreted carefully since it is not known whether the stimulation protocols were sufficient to increase NO levels in the muscle. To our knowledge, our present data is the first to show that blocking the increase in contraction-stimulated NOS activity by L-NMMA does not

affect the normal increase in glucose uptake during contraction. This dissociation between muscle NOS activity and glucose uptake during contractions has been supported by previously published data from our lab using nNOSμ KO mice (Hong *et al.*, 2015b; Hong *et al.*, 2016), which had at least normal contraction- and exercise-stimulated glucose uptake despite no measurable increase in muscle NOS activity. Taken together, these studies suggest that NOS activity is not required in the regulation of muscle glucose uptake during contraction.

We observed that peak muscle contraction forces and the decline in force with fatigue were similar to that of our previous studies (Figure 4.4) which indicates that the stimulatory conditions of the contracting muscle likely replicated that of our previous studies. The contraction-stimulated increase in glucose uptake above basal levels (~1.8 - 2-fold) from both of the contraction protocols used in this study are similar to two studies (Merry *et al.*, 2010b; Merry *et al.*, 2010c) while our most recent study reported a greater increase (~4-fold) (Hong *et al.*, 2015b). Importantly, contractions significantly increased NOS activity above basal levels, and L-NMMA, at an identical dose (100 μM) as used previously (Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b), was sufficient in blocking this increase. The ~4-fold increase in p-AMPK with contractions observed in this study is consistent with two of our previous studies (Merry *et al.*, 2010b; Merry *et al.*, 2010c), also suggests that we were able to closely replicate the contracting conditions of our previous work.

Although we aimed to closely follow the methodologies of our previous studies, subtle experimental differences do exist. This study used male C57BL/6 mice for inhibitor experiments, and while one previous study used the same mouse strain and sex sourced from the same vendor (Merry *et al.*, 2010b), the others have involved genetically modified mice (Merry *et al.*, 2010c; Hong *et al.*, 2015b), albeit backcrossed on a C57BL/6 background. Previous studies in rats by our group resulted in a NOS inhibition effect on glucose uptake during *in situ* contractions in one study using hooded Wistar rats (Ross *et al.*, 2007) but not in a subsequent study using Sprague-Dawley rats (Hong *et al.*, 2015a) raising the possibility that the role of NOS in regulating glucose uptake is strain-dependent. Furthermore, there are published studies reporting that substrain differences in rats have confounded experimental work of NOS-dependent vasodilation (Pollock & Rekito, 1998; Buhimschi *et al.*, 2001). In these studies, the same strain of rat maintained in two separate facilities over several generations resulted in different hypertensive responses to chronic L-NAME treatment. In the present study, although the C57BL/6 mice used were sourced from the same commercial vendor as in our earlier studies (Merry *et al.*, 2010b; Merry *et al.*, 2010c), which were undertaken nearly 10 years apart, it remains unclear whether such a factor has indeed confounded results of the present study.

It is important to note also that the present study was conducted in a different laboratory (Victoria University) than the three previous studies (The University of Melbourne) (Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b) and therefore it is possible that rodents were exposed to different environmental stimulus/conditions. Rearing

environment has been previously suggested to affect experimental results in C57BL/6 mice (Chang *et al.*, 2012), however, the C57BL/6 mice used across all our studies were purchased directly from the vendor with the experiments conducted within four weeks of arriving at the laboratory where experiments were conducted. Two previous studies using genetically modified mice (AMPK DN and nNOSμ KO mice) did however maintain their mice within the University of Melbourne laboratory where those experiments were conducted (Merry *et al.*, 2010c; Hong *et al.*, 2015b) and as such, those mice could have been exposed to different conditions for their entire life. Housing temperature, which is known to affect metabolic rate in mice (Abreu-Vieira *et al.*, 2015), was unlikely a factor in this study as facility temperatures were standard across all three of our studies. Other unknown environmental factors may have contributed to differences in our results but are difficult to delineate. Nonetheless, the importance of NO as a regulatory factor in muscle glucose uptake during contractions would be questionable if the conditions were to be so narrow.

Results from our NOS knockout experiments (Figure 4.6) provide further support that NOS is not a required mediator for contraction-stimulated glucose uptake. Consistent with previous observations (Hong *et al.*, 2015b), muscles lacking the predominant NOS isoform nNOSμ, had normal contraction-stimulated glucose uptake. Of note, however, in that study, L-NMMA was found to attenuate contraction-stimulated glucose uptake in both wildtype control mice and nNOSμ KO mice despite no detectable increase in NOS activity with contractions (Hong *et al.*, 2015b). In that study, only eNOS was detected in muscles from nNOSμ KO mice, suggesting that the L-NMMA effect was due to its action on the eNOS

isoform. One previous study reported that eNOS KO mice actually have higher, rather than lower muscle glucose uptake during treadmill running (Lee-Young *et al.*, 2010). However, the authors of that study acknowledged that the loss of eNOS probably resulted in exercise-induced muscle hypoxia which could have stimulated glucose uptake and masked any potential effect of the lack of eNOS. We therefore examined muscles from eNOS KO mice in the isolated muscle contraction model whereby the potential effects of blood flow are removed, with contraction-stimulated glucose uptake being similar between eNOS KO and control mice. Although it could be argued that single NOS isoform deletion may not necessarily result in a phenotype due to the compensatory persistence of remaining isoform(s) (Huang *et al.*, 2002; Hurt *et al.*, 2006), the lack of effect of NOS isoform deletion on glucose uptake is supported by the NOS inhibition data.

Although these results cast doubt on the role of NO/NOS on skeletal muscle glucose uptake during contractions, it is important to note that studies in humans appear to show a role of NO/NOS during exercise (Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Mortensen *et al.*, 2007). However, more work is required to examine the underlying mechanisms involved. Indeed, published human studies have not yet reported on whether skeletal muscle signalling or microvascular flow changes occur with NOS inhibitors during exercise.

In summary, we found that skeletal muscle glucose uptake in contracting muscles *ex vivo* is regulated by Rac1 but not NOS. Using pharmacological inhibitors, findings suggest that during contraction, activation of Rac1 does not require NOS activity and vice versa.

Surprisingly, the lack of NOS inhibitor effect on contraction-stimulated glucose uptake is in contrast to previous work from our group despite using a similar experimental model. Therefore, the findings of this study challenge the hypothesis that NO is an important regulator of muscle glucose uptake during contraction in mice.

CHAPTER FIVE: INVESTIGATION OF RHOGDI AS AN UPSTREAM REGULATOR OF RAC1 AND GLUCOSE UPTAKE DURING EXERCISE

5.1. Introduction

Elevated fasting blood glucose is a characteristic of type 2 diabetes due in part to the inability of insulin to effectively stimulate glucose uptake in peripheral tissues. Skeletal muscle accounts for the majority of total blood glucose disposal under euglycemic hyperinsulinemic conditions (DeFronzo *et al.*, 1985). Like insulin, exercise is also a potent physiological regulator of muscle glucose uptake, however, the signalling pathway activated by exercise is at least proximally distinct to insulin signalling (Richter & Hargreaves, 2013). Interestingly, the ability of exercise to stimulate muscle glucose uptake is maintained in insulin-resistant muscle (Kennedy *et al.*, 1999; Kingwell *et al.*, 2002) indicating that this signalling pathway remains intact despite defects in insulin signalling. Furthermore, rodent and human studies have demonstrated that exercise enhances muscle insulin sensitivity in the period following exercise (Wojtaszewski *et al.*, 2000a; Sjoberg *et al.*, 2017). Despite evidence for the benefits of exercise, many people with elevated blood glucose levels do not adhere to regular exercise programs (Praet & van Loon, 2009), and therefore other strategies are required. Understanding how exercise regulates glucose uptake could provide important

information for the development of drug therapies that lower blood glucose levels by targeting this signalling pathway.

There is strong evidence that the small Rho family GTPase Rac1 (Ras-related C3 botulinum toxin substrate 1) plays an essential role in the regulation of muscle glucose uptake during exercise. Indeed, muscle Rac1 is activated in rodents during stretching, contraction and exercise (Sylow *et al.*, 2013b; Sylow *et al.*, 2015) and in humans during exercise (Sylow *et al.*, 2013b). Initial cell culture experiments reported that constitutively activated Rac1 stimulated GLUT4 translocation in L6 myotubes (Ueda *et al.*, 2008). In a more recent series of studies, Sylow and colleagues demonstrated that Rac1 knockout or pharmacological inhibition of Rac1 attenuates muscle glucose uptake during passive stretching (Sylow *et al.*, 2015), contractions (Sylow *et al.*, 2013b; Sylow *et al.*, 2017b), and exercise (Sylow *et al.*, 2016; Sylow *et al.*, 2017b).

To date, studies investigating how Rac1 regulates muscle glucose uptake in mature muscle models during contractile activity have mostly examined Rac1-downstream effectors such as p21 -activated kinases 1 and 2 (PAK1/2). For example, Rac1 knockout mice have less of an increase in PAK1/2 phosphorylation with stretch (Sylow *et al.*, 2015) and contraction (Sylow *et al.*, 2013b). There has been limited examination of what the upstream regulators of Rac1 are. In cultured muscle cells, activation of AMP-activated kinase (AMPK) by AICAR was shown to stimulate Rac1 activity (Lee *et al.*, 2008) suggesting AMPK acts as an upstream regulator of Rac1. However, more recent studies do not link AMPK with

Rac1 activity. Indeed, Rac1 activity was not increased in the presence of AICAR in C2C12 myotubes (Sylow *et al.*, 2013b) and Rac1 signalling was not altered by either AICAR-stimulated AMPK activation or during treadmill running in mice overexpressing a kinase-dead form of AMPK (Sylow *et al.*, 2013b). Furthermore, passive stretching increases Rac1 activity (Sylow *et al.*, 2013b; Sylow *et al.*, 2015) without AMPK activation (Chambers *et al.*, 2009; Jensen *et al.*, 2014; Sylow *et al.*, 2015), suggesting that Rac1 activation does not also require AMPK activation. In another study, mice lacking functional mTORC2 signalling have reduced exercise-stimulated glucose uptake but maintain normal Rac1 signalling (Kleinert *et al.*, 2017) indicating that mTORC2 does not function to regulate Rac1. There is evidence from cell culture studies that nitric oxide (NO) may act as an upstream regulator of Rac1 (Heo & Campbell, 2005; Godfrey & Schwarte, 2010), however, findings presented within this thesis question the involvement of NO in glucose uptake and suggest that during muscle stretching or contraction, Rac1 is activated independently of NO.

Rac1 functions as a molecular switch, mediated by guanine exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine dissociation inhibitors (GDIs). In brief, GEFs and GAPs turn the Rac1 system on and off whereas GDI's sequester Rac1 in an inactive complex (Cherfils & Zeghouf, 2013). The GDI which targets Rac1 is RhoGDI (DerMardirossian & Bokoch, 2005), and findings from cell culture studies suggest that RhoGDI is a negative regulator of Rac1 activity (Nishiyama *et al.*, 1994; Kowluru & Veluthakal, 2005). In one study, the induction of RhoGDI in KB cells (a subline of the ubiquitous KERATIN-forming tumour cell line HeLa) was reported to block Rac1-mediated

cytoskeletal remodelling (Nishiyama *et al.*, 1994). In another study, overexpression of RhoGDI was reported to reduce glucose-induced insulin secretion from isolated pancreatic β-cells (Kowluru & Veluthakal, 2005), a process which is known to involve Rac1 (Li *et al.*, 2004), whereas siRNA-mediated knockdown of RhoGDI increased glucose-induced insulin secretion (Kowluru & Veluthakal, 2005). Interestingly, preliminary findings from Lykke Sylow's group in Denmark report that siRNA-mediated knockdown of the RhoGDI isoform α (RhoGDI α) in muscle cells resulted in an increase in Rac1 activity and increased insulin-stimulated glucose uptake (Moller *et al.*, 2017). Therefore, RhoGDIα appears to be an important upstream regulator of Rac1 activity. However, this remains to be tested in the context of skeletal muscle glucose uptake during exercise.

Therefore, this study examined whether RhoGDIα regulates skeletal muscle glucose uptake during exercise and whether this occurs via modulation of the Rac1 signalling pathway. Systemic delivery of an rAAV6-RhoGDI vector was used to overexpress RhoGDI protein in skeletal muscle of mice. It was hypothesised that RhoGDIα overexpression would attenuate the normal increase in muscle glucose uptake during running exercise, presumably through reductions in Rac1 signalling.

5.2. Methods

5.2.1. Animals

Animal experimentation described within this chapter was conducted at the University of Copenhagen, Denmark, with the prior ethical approval of the Danish Animal Experiments Inspectorate and complied with the European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes. I travelled to the University of Copenhagen to participate in the mouse exercise testing as well as performing all muscle protein signalling analyses. This work was undertaken within the Section of Molecular Physiology, Department of Nutrition, Exercise and Sports at the University of Copenhagen. All mice were housed in standard rodent cages and maintained at ~21°C on a 12 hour dark/light cycle (exercise tests conducted in light cycle) with access to standard rodent chow and water *ad libitum.* Female C57BL/6NTac mice (aged 20-22 weeks) were used for this study. Female mice were previously used for Rac1 inhibitor and Rac1 KO published studies (Sylow *et al.*, 2013b; Sylow *et al.*, 2016; Sylow *et al.*, 2017a; Sylow *et al.*, 2017b). Mice overexpressing muscle RhoGDI protein were generated and maintained at the University of Copenhagen, Denmark by Dr Lykke Sylow and Lisbeth Moller. Mice received a tail-vein injection of recombinant adeno-associated virus serotype-6-RhoGDIα (rAAV6-RhoGDIα) or empty control vector (herein referred to as either RhoGDI or control mice) 8 weeks before exercise test. Transduction of rAAV-6 in skeletal muscle by intravenous delivery has been well documented to target skeletal muscle with robust results (Gregorevic

et al., 2004; Percival *et al.*, 2007; Zincarelli *et al.*, 2008).The study involved exercising and sedentary mice, and all mice underwent a maximal exercise capacity test prior to the glucose uptake exercise test.

5.2.2. Maximal exercise capacity test

All mice were acclimatised to the enclosed treadmill on one occasion one week prior to the maximal exercise capacity test. Acclimatisation consisted of 3 x 5 min at 10 m/min and 2 x 5 min at 16 m/min running on a 0° incline treadmill. The maximal exercise test was conducted on a treadmill at 10° incline and consisted of a 5 min warm-up at 10 m/min, and then the speed was increased by ~1.2 m/min every minute until exhaustion. A compressed air gun delivering short bursts of air from the back of the treadmill was used to encourage the mice to run during the acclimatisation, maximal capacity, and exercise testing. Exhaustion was defined as the point at which mice remained on the back of the treadmill despite multiple short bursts of compressed air directed behind them. This point was determined by a researcher who was blinded to the experimental groups.

5.2.3. Exercise test

Mice were allocated into treatment groups as follows: WT control group, 2 and 6 for rest and exercise; RhoGDI group, 3 and 5 for rest and exercise. Given the variability in glucose uptake and muscle signalling with exercise, more mice were allocated to the exercising groups than the resting control groups. The exercise test was conducted six days

after the maximal exercise test. All mice exercised for 20 min on a treadmill at 10° incline at a speed of 0.29 m/s. This corresponded to approximately 60% of the maximal running capacity of both groups (control mice 0.50 ± 0.02 m/s; Rho GDI mice 0.49 ± 0.02 m/s). No statistical difference between groups for maximal running speed was found. Sedentary mice were allowed to move freely on a stationary treadmill for the 20 min test. In all mice, immediately prior to the onset of the exercise test, a saline bolus containing 0.1 mM 2-deoxyglucose and 50 µCi 2-[^{3}H]-deoxyglucose ([^{3}H]-2DG) was injected intraperitoneally to enable measurement of muscle-specific glucose uptake (Sylow *et al.*, 2017b). Blood samples were collected via the tail vein before and after the exercise and analysed for glucose concentration. Immediately following exercise or rest, a plasma sample was collected to measure specific [^{3}H]-2DG tracer activity. This method to estimate muscle glucose uptake during exercise has been previously published (Sylow *et al.*, 2016; Kleinert *et al.*, 2017; Sylow *et al.*, 2017b; Henríquez-Olguin *et al.*, 2019). At the cessation of the exercise test, mice were killed by cervical dislocation and quadriceps, gastrocnemius, and soleus muscles rapidly harvested and snap-frozen in liquid nitrogen.

5.2.4. Muscle homogenisation

Muscle tissue was crushed under liquid nitrogen and then homogenised by a steel-bead for 30 s (30 Hz) (TissueLyser, Qiagen) in ice-cold buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM sodium orthovanadate, 2 mM EDTA, 1% NP-40, 10% glycerol, 2 mM

phenylmethanesulfonyl fluoride, 1 mM MgCl2, 1 mM CaCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 3 mM benzamidine), and then rotated end-over-end for 30 min at 4°C. Following centrifugation (10,000 g) for 15 min at 4°C, lysate supernatants were collected and protein concentrations determined using the BCA method (Pierce). Lysates were diluted to a standard concentration (1 μg/μL) in loading buffer and heated at 96°C for 3 min before being frozen at -20°C for future analysis.

5.2.5. Immunoblotting

Total protein (12-15 μg) was separated by SDS-PAGE using self-cast gels (7% or 11% gels) and semi-dry transferred (Turbo blot system, BioRad, CA, USA) to PVDF membranes (Immobilon Transfer Membrane, Millipore). Membranes were blocked for 30 min at room temperature in Tris-buffered saline-Tween 20 (TBST) containing either 2% skim milk or 3-5% BSA. The dilution of skim milk and BSA used for blocking and antibody dilution have been previously optimised within the laboratory group (University of Copenhagen). Membranes were then incubated in primary antibody overnight at 4°C. The primary antibodies used were: Rac1 (Cytoskeleton Inc, CO, USA); RhoGDI, RhoA, AMPKα2, p-AMPK^{Thr172}, p-ACC2^{Ser212}, p-PAK1/2$^{Thr423/402}$, p-p38 MAPK$^{Thr180/182}$ (Cell Signalling Technology, MA, USA); ACC2 (gift from G. Hardie, University of Dundee); p-TBC1D1^{Ser231} (Millipore, MA, USA); and GLUT4 (Thermo Scientific, IL, USA). Following overnight incubation, membranes were washed in TBST and then incubated in horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. Protein bands

were visualised using Bio-Rad ChemiDoc imaging system and enhanced chemiluminesence substrate (ECL+, Amersham Biosciences), and quantified using ImageLab software (Bio-Rad). The immunoblotting work performed in this study was undertaken in a different laboratory (University of Copenhagen, Denmark) to that of Chapter Three and Chapter Four, and the protocol undertaken did not involve normalising protein bands to a loading control using stain-free gels. Instead, Coomassie brilliant blue stained membranes were used to confirm that protein loading was similar across samples (presented in Appendix B).

5.2.6. Muscle glucose uptake and glycogen measurements

Muscle glucose uptake was estimated by measuring the muscle accumulation of [^{3}H]-2DG-phosphate ([^{3}H]-2DG-P) via the precipitation method using 0.1 M $Ba(OH)_2$ and 0.1 M $ZnSO_4$ as previously described (Fueger *et al.*, 2004; Sylow *et al.*, 2016; Sylow *et al.*, 2017b). Muscle [^{3}H]-2DG-P activity was divided by the circulating plasma [^{3}H]-2DG activity and multiplied by plasma glucose concentration to calculate muscle glucose uptake expressed as µmol/g/hr. Muscle glycogen content was measured as glycosyl units after acid hydrolysis and was determined fluorometrically (Passonneau *et al.*, 1967; Sylow *et al.*, 2016).

5.2.7. Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0 software. A two-factor (treatment and genotype) ANOVA, or Student's t-test were used. The significance level was set at $P < 0.05$.

5.3. Results

5.3.1. Transduction efficiency of systemic rAAV6 delivery

Systemic delivery of rAAV6-RhoGDIα significantly increased RhoGDIα expression in quadriceps (~2.5-fold) and gastrocnemius muscles (~3.5-fold) ($P < 0.001$). In contrast, RhoGDIα expression in soleus muscles was similar between mice injected with rAAV6-RhoGDIα or control (Figure 5.1) indicating poor transduction efficiency in this muscle. Interestingly, in soleus muscle, expression of the FLAG-tagged RhoGDIα was detected in some but not all mice injected with the rAAV6-RhoGDIα vector (Figure 5.2). The secondary protein band detected above the endogenous RhoGDIα band is due to the additional molecular mass of the FLAG tag and both bands were analysed.

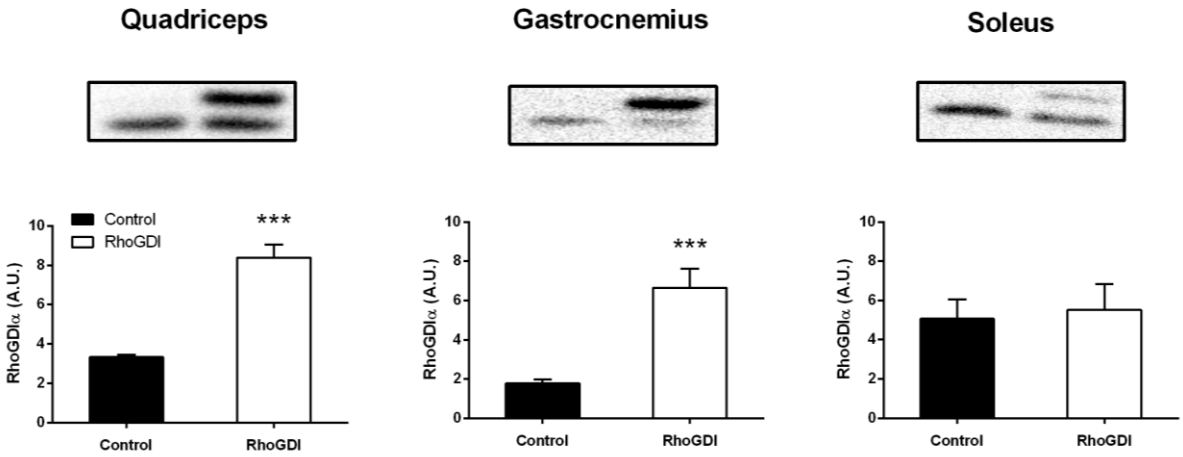

Figure 5.1. RhoGDIα expression. Quantification and representative blots showing RhoGDIα protein expression in quadriceps, gastrocnemius, and soleus muscles of AAV-RhoGDI and control mice (n = 7-8 per group). Data are means ± SEM. *** $P < 0.001$ vs control group.

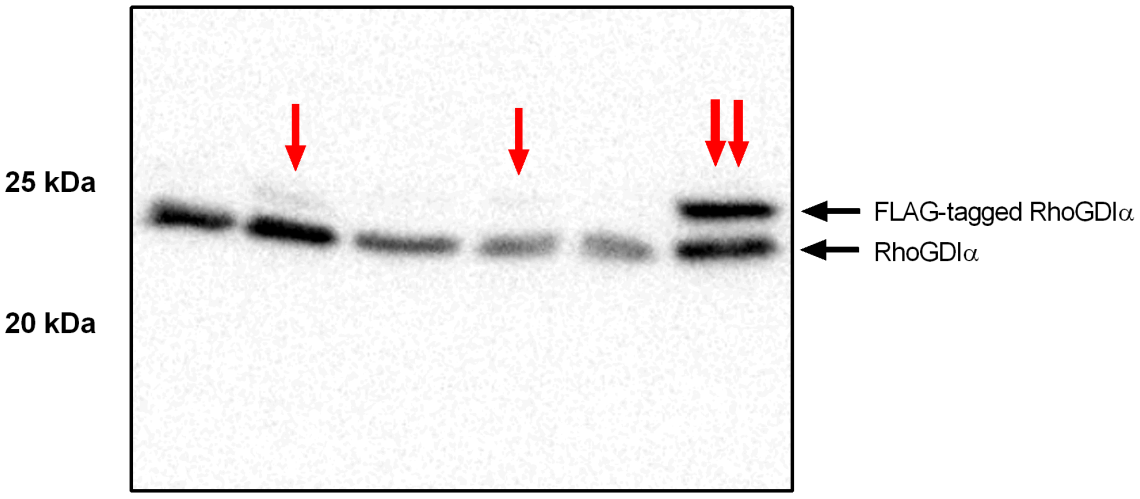

Figure 5.2. Variability of rAAV6-RhoGDIα transduction in soleus muscle. Representative immunoblot using anti-RhoGDIα antibody. Lanes 1, 3, and 5 are samples from mice injected with an empty vector (control). Lanes 2, 4, and 6 are samples from mice injected with rAAV6-RhoGDIα vector. Single vertical arrows denote samples showing minimal to no detectable expression of FLAG-tagged RhoGDIα. Double vertical arrows denote FLAG-tagged RhoGDIα indicating robust overexpression of RhoGDIα following systemic delivery of rAAV6-RhoGDIα vector.

5.3.2. RhoGDIα overexpression increases Rac1 and RhoA protein content

Rac1 content was enhanced approximately 2-fold in quadriceps and approximately 1.5-fold in gastrocnemius muscles of RhoGDI mice ($P < 0.001$ and < 0.01 respectively), whereas Rac1 expression was similar between RhoGDI and control mice in soleus muscles. Likewise, expression of the Rho GTPase RhoA was significantly increased approximately 3-fold in quadriceps muscle and approximately 2-fold in gastrocnemius muscles ($P < 0.01$ and 0.05 respectively), but not soleus muscles (Figure 5.3). This was consistent with the RhoGDIα protein expression (Figure 5.1). Since the rAAV6-RhoGDIα delivery only increased RhoGDIα expression in quadriceps and gastrocnemius muscles, analysis of muscle glucose uptake, glycogen content, and protein phosphorylation with exercise was only conducted using these muscles and not the soleus which displayed no overall increase in RhoGDIα expression.

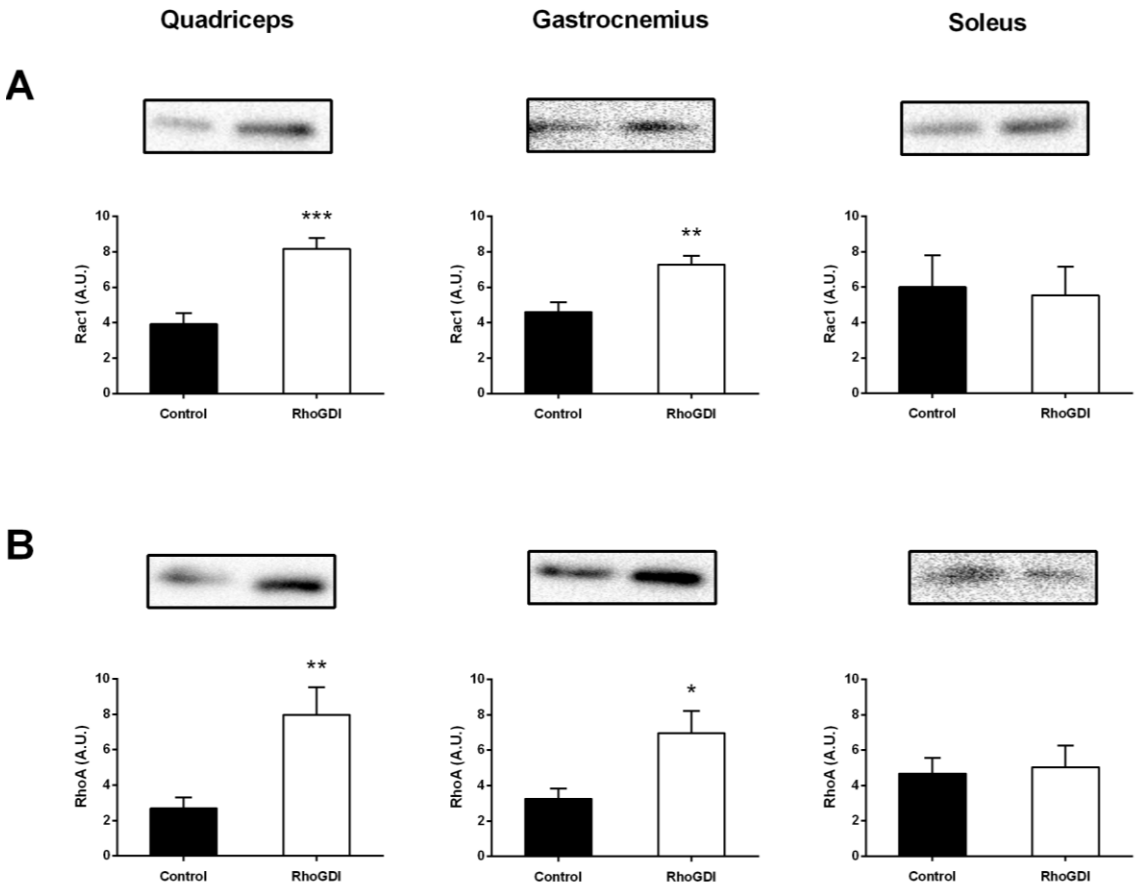

Figure 5.3. Expression of RhoGTPase proteins. Quantifications and representative blots showing protein expression of A) Rac1, and B) RhoA in gastrocnemius, quadriceps, and soleus muscles of RhoGDI and control mice (n = 7-8 per group). Data are means ± SEM. */**/*** P < 0.05/0.01/0.001 vs control group (t-test). A.U., Arbitrary unit.

5.3.3. GLUT4 protein

GLUT4 protein content was similar between control and RhoGDI mice in both quadriceps and gastrocnemius muscles (Figure 5.4).

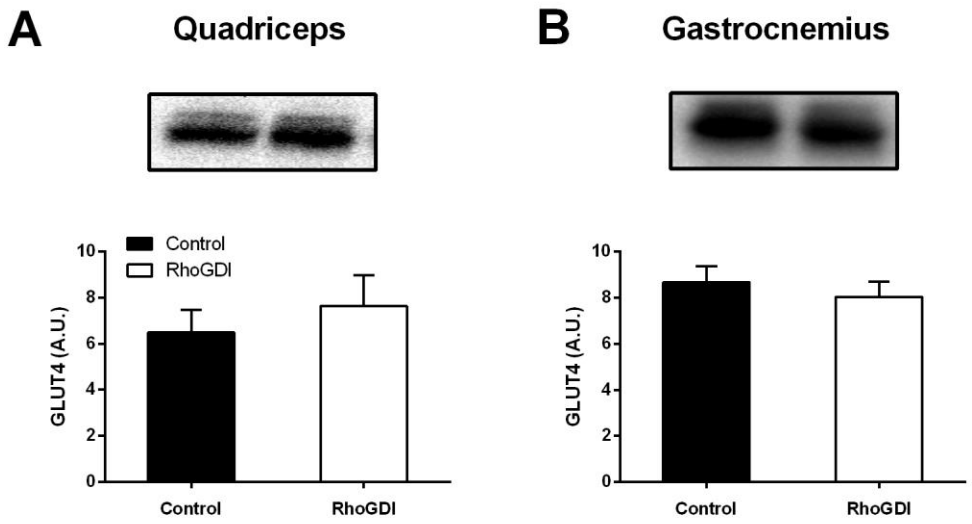

Figure 5.4. GLUT4 protein expression. Quantifications and representative blots showing GLUT4 protein expression in quadriceps and gastrocnemius muscles of control and RhoGDI and control mice (n = 7-8 per group). Data are means ± SEM. A.U., Arbitrary unit.

5.3.4. Body mass, exercise capacity, and blood glucose levels

Body mass was similar between control and RhoGDI mice (21.1 ± 0.5 and 21.5 ± 0.4 g). The maximal running speed achieved during the exercise capacity test was similar between control and RhoGDI mice (0.50 ± 0.02 and 0.49 ± 0.02 m/s). Blood glucose was approximately 1.5 mM higher at both rest and at the end of exercise in RhoGDI mice compared with control mice (6.8 ± 0.3 and 8.6 ± 0.2 mmol for control mice; 8.1 ± 0.5 and 10.3 ± 0.8 mmol for RhoGDI mice, respectively $P < 0.01$) (Figure 5.5).

5.3.5. RhoGDIα overexpression does not alter exercise-mediated muscle glucose uptake

Exercise significantly increased glucose uptake in both quadriceps and gastrocnemius muscles (Figure 5.6) and this was not statistically different between genotypes in either muscle.

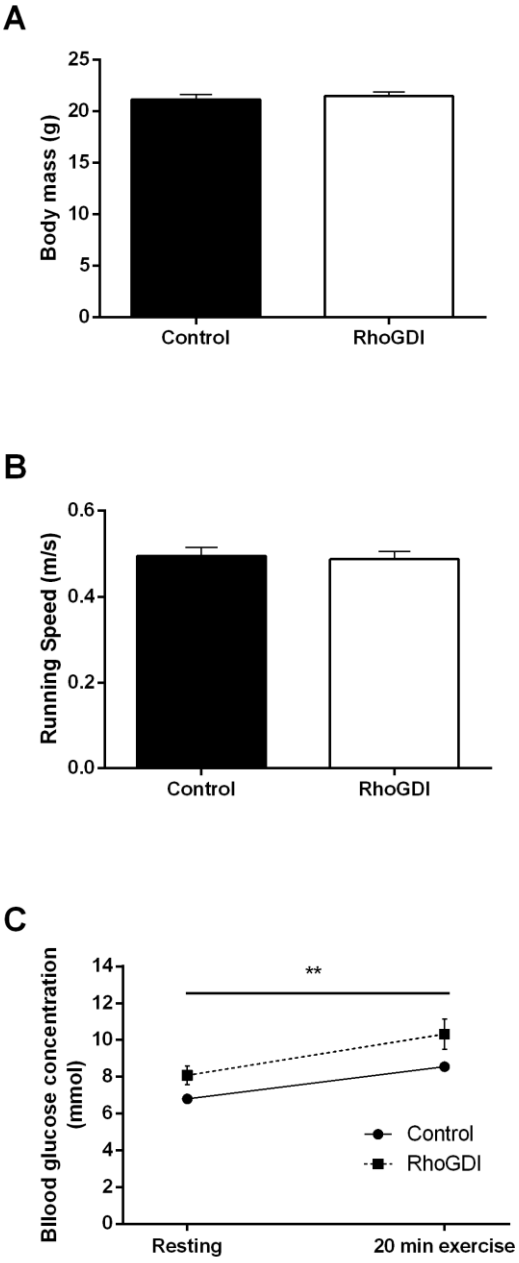

Figure 5.5 (Continued on the following page)

Figure 5.5 Body mass, exercise capacity, and blood glucose levels. Bar graph showing A) body mass, B) maximal running speed, and C) blood glucose levels at rest and after 20 min running exercise at 60% max treadmill running speed between control and RhoGDI mice (n = 8 per group). Data are means ± SEM. ** $P < 0.01$ main effect for RhoGDI overexpression (ANOVA).

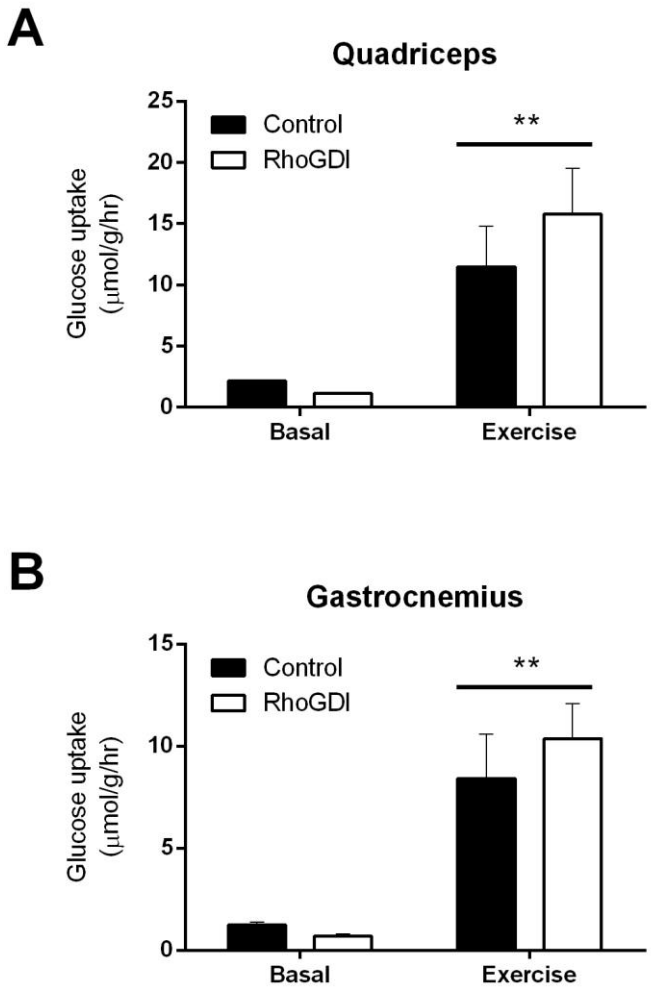

Figure 5.6 Muscle glucose uptake during exercise. 2-deoxyglucose uptake at rest (Basal) and during 60% max treadmill running speed (Exercise) in A) Quadriceps muscle and B) Gastrocnemius muscle of control and RhoGDI overexpressing mice (n = 2/3 for basal and 6/5 for exercise per group). Data are means ± SEM. ** $P < 0.05$ main effect of exercise (ANOVA).

5.3.6. Muscle glycogen

Muscle glycogen content tended to decrease with exercise in quadriceps muscles (P = 0.06) and in both control and RhoGDI mice. In gastrocnemius muscles, glycogen content was not significantly altered by exercise (P = 0.15) (Figure 5.7).

5.3.7. Exercise-stimulated protein signalling

Because directly measuring Rac1 activity in mature muscle is difficult phosphorylation of the downstream Rac1 kinase PAK1 was used as a surrogate for Rac1 activation (Sylow *et al.*, 2013a; Sylow *et al.*, 2013b). In quadriceps muscle, exercise tended to increase p-PAK1^{Thr423} (~25%) above basal levels (P = 0.09) and this was similar between control and RhoGDI mice. Exercise failed to increase PAK1 phosphorylation in gastrocnemius muscles in both groups (Figure 5.8).

In quadriceps muscle, exercise increased phosphorylation of AMPK^{Thr172} approximately two-fold in both groups ($P < 0.05$) while in the gastrocnemius, there was a tendency p-AMPK to be increased with exercise (P = 0.05). Phosphorylation of the downstream AMPK target ACC^{Ser212} increased with exercise in quadriceps muscle approximately 2-fold ($P < 0.05$), and this was similar between control and RhoGDI mice. In gastrocnemius muscle p-ACC^{Ser212} was not statistically different between rest and exercise in both genotypes (P = 0.15) (Figure 5.9). Phosphorylation of TBC1D1^{Ser321} a target of AMPK signalling was examined in the quadriceps muscle, given that p-AMPK was

significantly increased in this muscle by exercise. p-TBC1D1^{Ser231} tended to increase with exercise in the quadriceps muscle (P = 0.07) (Figure 5.10). This protein was not measured in gastrocnemius.

p38 MAPK has also been implicated in regulating glucose uptake during mechanical loading (Chambers *et al.*, 2009) although its role in contraction-stimulated glucose uptake is not clear (Somwar *et al.*, 2000; Jensen *et al.*, 2014). Phosphorylated p38 MAPK$^{Thr182/Tyr180}$ was significantly increased by exercise in the quadriceps muscle (Figure 5.11) (P < 0.05) and this was not statistically different between control and RhoGDI mice. In the gastrocnemius muscle, p-p38 MAPK$^{Thr182/180}$ was similar between rest and exercise levels.

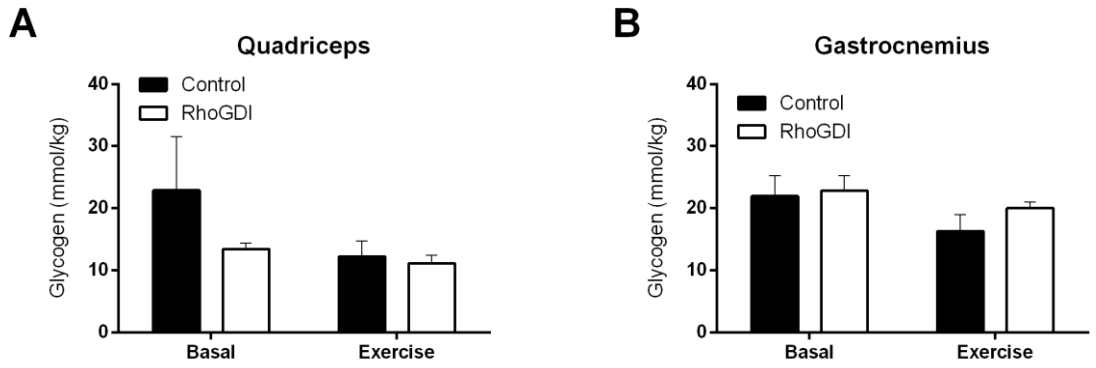

Figure 5.7 Muscle glycogen content. Muscle glycogen content at rest (Basal) and immediately following 60% max treadmill running speed (Exercise) in A) Quadriceps muscle and B) Gastrocnemius muscle of control and RhoGDI overexpressing mice (n = 2/3 for basal and 6/5 for exercise per group). Data are means ± SEM. P = 0.06 main effect of exercise for Quadriceps muscle. No significant difference in Gastrocnemius muscle (ANOVA).

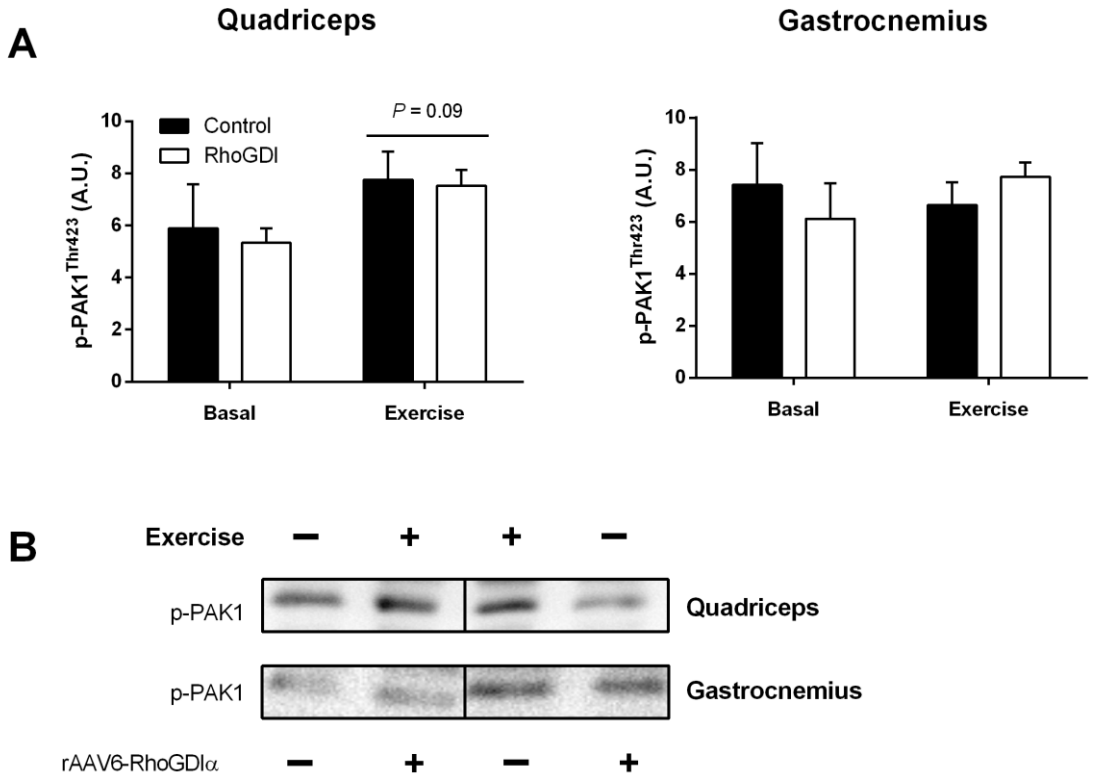

Figure 5.8 PAK1 phosphorylation. A) Quantifications, and B) representative blots of p-PAK1^{Thr423} in quadriceps and gastrocnemius muscles at rest (Basal) immediately following 60% max treadmill running speed (Exercise) (n = 2/3 basal and 6/5 exercise) in control and RhoGDI mice. Data are means ± SEM. P = 0.09 main effect of exercise (ANOVA). A.U., Arbitrary unit.

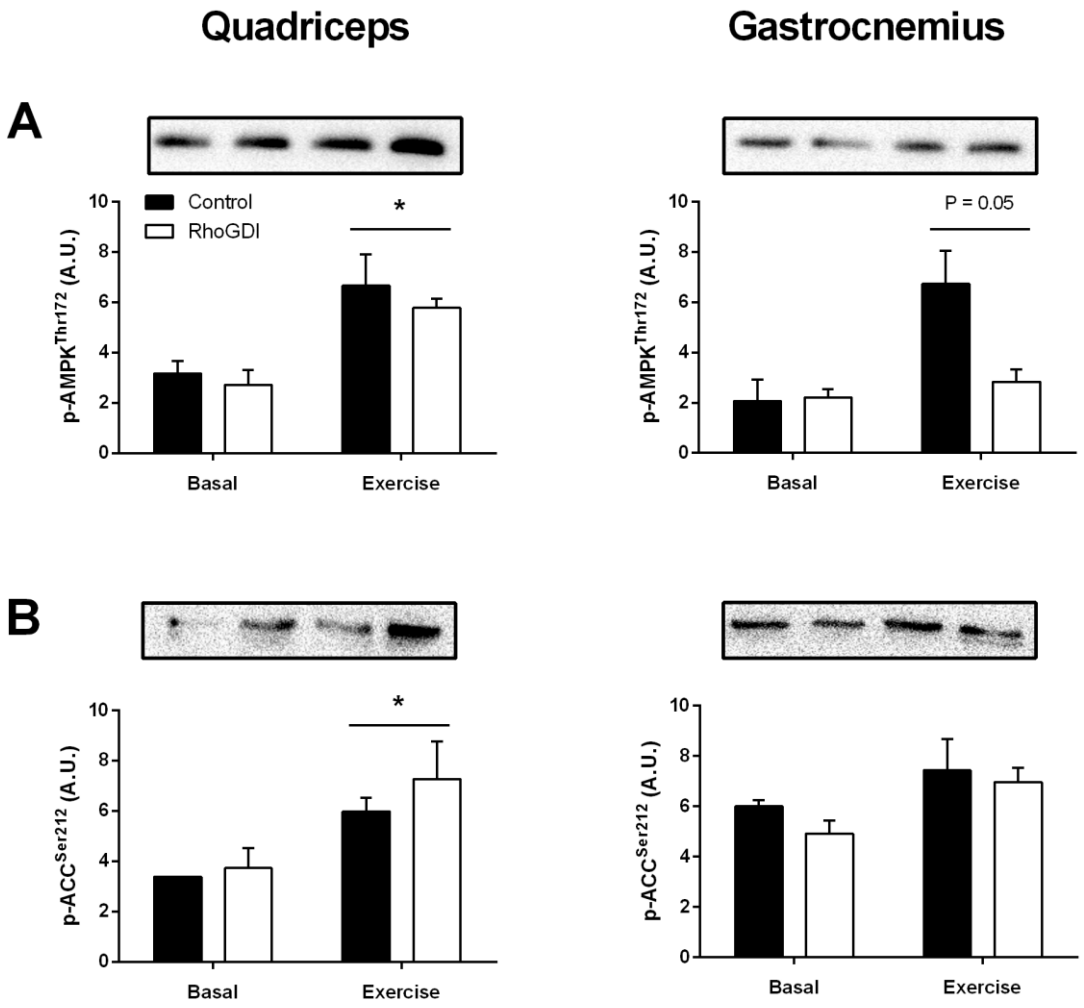

Figure 5.9 AMPK signalling. Quantifications and representative blots showing A) p-AMPK^{Thr172}, and B) p-ACC^{Ser212} in quadriceps and gastrocnemius muscles of control and RhoGDI mice at rest (Basal) and immediately following 60% max treadmill running speed (Exercise) (n = 2/3 basal and 6/5 exercise). Data are means ± SEM. * $P < 0.05$ main effect of exercise for Quadriceps, P = 0.05 main effect of exercise in Gastrocnemius for p-AMPK (ANOVA). A.U., Arbitrary unit.

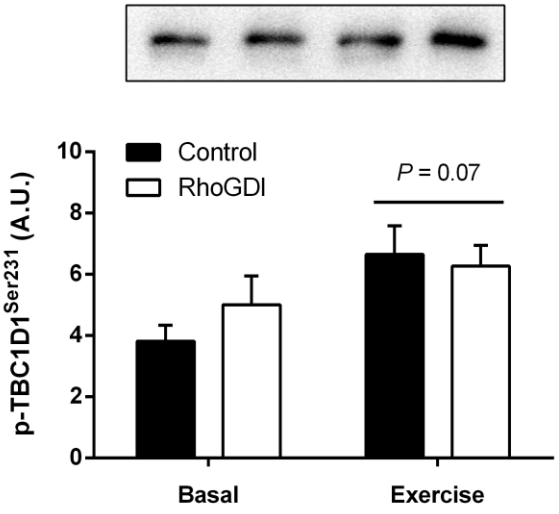

Figure 5.10 p-TBC1D1^{Ser231} during exercise. Quantification and representative blot showing phosphorylation status of TBC1D1^{Ser231} at rest (Basal) and immediately following 60% max treadmill running speed (Exercise) in quadriceps muscles of control and RhoGDI mice (n = 2/3 basal and 6/5 exercise). Data are means ± SEM. P = 0.07 main effect of exercise (ANOVA). A.U., Arbitrary unit.

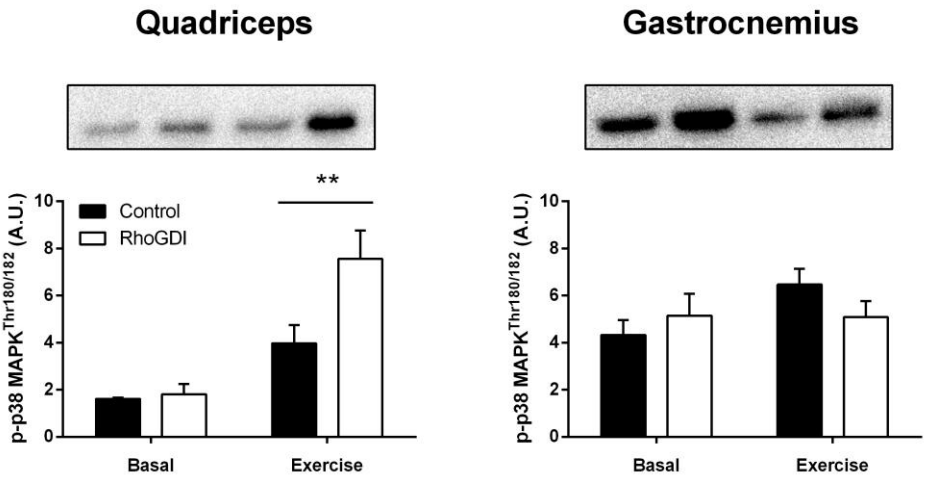

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Figure 5.11 p-p38 MAPK$^{Thr180/Tyr182}$. Quantification and representative blots showing p-p38 MAPKThr180/Tyr182 quadriceps and gastrocnemius muscles at rest (Basal) and immediately following 60% max treadmill running speed (Exercise) in control and RhoGDI mice (n = 2/3 basal and 6/5 exercise). Data are means ± SEM. ** $P < 0.01$ exercise main effect (ANOVA). A.U., Arbitrary unit.

5.4. Discussion

The main finding was that overexpression of RhoGDIα in muscle did not appear to attenuate muscle glucose uptake during treadmill running. RhoGDIα overexpression was accompanied by significant increases in the expression of the RhoGTPase proteins Rac1 and RhoA. We found that exercise tended to increase phosphorylation of the Rac1 downstream target PAK1 and this was similar in RhoGDIα overexpressing mice, which may suggest that Rac1 activity was not altered. However, the compensatory increase in Rac1 with RhoGDI overexpression makes the Rac1 signalling data difficult to interpret.

RhoGDIα is suggested to negatively regulate Rac1 activity (Garcia-Mata *et al.*, 2011). Rac1 activity is difficult to measure in mature muscle using commercial assay kits (L. Sylow, University of Copenhagen, *personal communication*) and instead, PAK1 phosphorylation, was used as a surrogate readout of Rac1 activity in this study (Sylow *et al.*, 2013b; Sylow *et al.*, 2015). At rest, p-PAK1 was similar between RhoGDI overexpressing muscles and control muscles suggesting that RhoGDI did not inhibit basal Rac1 activity. The lack of effect of RhoGDIα on basal Rac1 signalling in this study is in contrast to other studies which used cell culture models. Indeed, one study found that RhoGDI overexpression reduced Rac1 activity (Nishiyama *et al.*, 1994) while other studies (Bozza *et al.*, 2015; Moller *et al.*, 2017) report that knockdown of RhoGDI increased Rac1 activation.

Given that one of the functions of RhoGDIα is to prevent the Rho GTPase protein from being converted to a GTP-bound active conformation and subsequent interaction with

effectors (Garcia-Mata *et al.*, 2011), it was expected in this study that overexpression of RhoGDIα would blunt the increase in exercise-stimulated Rac1 activity in this study. However, exercise-stimulated p-PAK1 was similar between RhoGDI and control mice, suggesting that Rac1 activation was not altered by the increase in RhoGDI. The observation that muscle glucose uptake during exercise was also similar between RhoGDI and control mice fits with this lack of effect of RhoGDI overexpression on Rac1 signalling.

In a previous study, it was reported that depletion of RhoGDI in eukaryotic cells resulted in a marked reduction in Rac1 content, however, the relative amount of active Rac1 was significantly elevated thereby ensuring that absolute Rac1 activity was not reduced (Boulter *et al.*, 2010). In the present study, it would be worth considering whether changes in the amount of active Rac1 occurred to maintain this signalling pathway. However, since we did not measure Rac1-GTP content, we are unable to determine if such differences occurred. Nonetheless, the compensatory up-regulation of Rac1 protein content after RhoGDIα overexpression may serve as an example of flexibility in the Rac1 signalling pathway in order to maintain glucose uptake regulation during muscle contraction. The maintenance of this signalling pathway suggests that Rac1 signalling may be a critical regulator of glucose uptake. Indeed, it has previously been shown that muscle glucose uptake during exercise is attenuated in Rac1 KO mice by 60-80% in gastrocnemius and quadriceps muscles, and completely blocked in soleus muscles (Sylow *et al.*, 2016).

Whether the exercise model used in this study was sufficient for probing the RhoGDI-Rac1-PAK1 signalling mechanism should be given consideration. Multiple signalling proteins have been implicated in the regulation of muscle glucose uptake during exercise (Richter & Hargreaves, 2013), and it is likely that the relative contribution of the signalling factors involved is dependent on the intensity of the contractile conditions (Jensen *et al.*, 2014). Indeed, previous findings suggest that Rac1 activation in muscle exhibits a dose-response pattern with respect to exercise intensity. In mouse soleus muscle after 30 minutes of treadmill running at 50% and 70% of maximum running speed, Rac1-GTP binding was reported to increase by approximately 50% and 100% respectively. This was mirrored by increases in p-PAK1 by approximately 60% and 100% (Sylow *et al.*, 2013b). This also fits with a previous observation that the reduction in quadriceps glucose uptake in Rac1 KO mice uptake was greater at 85% maximal running capacity (~50% reduction) compared with 65% maximal running capacity (~30% reduction) (Sylow *et al.*, 2016). The exercise intensity used in the current study (60%) was less than that of previous studies (65-85%) where Rac1-deficiency has been shown to attenuate muscle glucose uptake (Sylow *et al.*, 2016; Sylow *et al.*, 2017b). In this study, only modest increases in p-PAK1 in quadriceps muscles ($P = 0.09$) were observed, with no increase in gastrocnemius muscle. Further experimental work should be carried out to examine whether RhoGDIα overexpression perturbs Rac1 signalling at higher exercising intensities whereby greater levels of Rac1/PAK signalling activation occurs (Sylow *et al.*, 2013b).

Binding of RhoGTPase proteins to RhoGDI may serve to stabilise and protect the GTPase from degradation. A previous study has shown that depletion of RhoGDI in HeLa cells via transfection of siRNA RhoGDI resulted in a decreased level of Rho proteins (Boulter *et al.*, 2010). Consistent with this function, in this study overexpression of RhoGDI in quadriceps and gastrocnemius muscles markedly increased Rac1 and RhoA protein expression. In contrast however, these proteins were not upregulated in the soleus muscle which exhibited no increase in RhoGDIα expression by rAAV6-RhoGDIα delivery (Figure 5.1). Intriguingly, expression of the FLAG-tagged RhoGDIα as verified by immunoblot showed considerable variability between samples, with substantial expression of the vector in some mice, but completely absent in others. A previous study reported that the transduction of rAAV6 in soleus required a higher dose than in EDL muscle, and the authors noted a degree of variability from injection to injection when lower doses were used (Blankinship *et al.*, 2004). Therefore, the variability, and overall lack of significant increase in RhoGDIα protein in the soleus observed in the current study could suggest that an insufficient vector dose was used for the soleus.

The RhoGDI isoform α investigated in this study, is known to bind several GTPases including the small GTPase RhoA (Michaelson *et al.*, 2001). Similar to the observed Rac1 upregulation in the RhoGDIα over-expressing mice, an increase in RhoA protein content was also found (Figure 5.3). RhoA is known to be involved in many cellular processes such as protein synthesis, gene transcription, and regulation of the cell cytoskeleton (Ridley & Hall, 1992). Since the cytoskeleton is considered an important component of the signal

transduction pathway regulating glucose uptake during muscle contractions (Sylow *et al.*, 2013b; Sylow *et al.*, 2015), it is worth considering whether RhoA serves an overlapping function with Rac1 in this regard. However, no role for RhoA in the regulation of skeletal muscle glucose uptake during contractions or exercise has been established.

Mechanical stress in the form of passive stretching has been shown to activate Rac1 signalling (Sylow *et al.*, 2015) and as such, upstream regulators of Rac1 are also likely part of the mechanical stress-activated signalling pathway. Passive stretching has been shown to increase NO production in cells (Zhang *et al.*, 2004; Wozniak & Anderson, 2009) and isolated mature muscle (Tidball *et al.*, 1998) and NO produced by NOS enzymes have been shown to regulate contraction- and exercise-stimulated glucose uptake (Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Merry *et al.*, 2010b; Hong *et al.*, 2015b). Furthermore, treatment of C2C12 myotubes with a NO donor was shown to activate Rac1/PAK1 signalling (Godfrey & Schwarte, 2010), placing NOS as a potential upstream regulator of Rac1-dependent glucose uptake. However, the role of NOS as an upstream regulator of Rac1/PAK1 signalling in the context of glucose uptake is unlikely given the results of this thesis which show that Rac1 signalling does not require NOS activation (See Chapter Three and Chapter Four).

In this study, the interpretation of Rac1 activity was based on the phosphorylation status of PAK1, and it is acknowledged that this is not as precise as measuring Rac1-GTP directly. Furthermore, it is possible that Rac1 activity during exercise is transient (Stierwalt *et al.*, 2018) and this is not captured by a measure of its downstream kinase PAK1 at one

time-point (end of exercise). PAK1 has long been considered a downstream target of Rac1 and assumed to play a role in the regulation of exercise-stimulated glucose uptake (Sylow *et al.*, 2013b). However, following the experimental work performed in this thesis, a recent study has questioned the role of PAK1 in glucose uptake (Moller *et al.*, 2019). In that study, PAK inhibitors were reported to attenuate contraction-stimulated glucose uptake, however, PAK1 KO mice had normal contraction-stimulated glucose uptake (Moller *et al.*, 2019). Therefore, although PAK1 signalling appears to mirror Rac1 activation during muscle contractions (Sylow *et al.*, 2013b) and has been used as a readout of Rac1 activation, its role in signalling towards glucose uptake is not clear.

It is acknowledged that the sample size examined in this study makes the interpretation of findings difficult. Because the role of RhoGDIα had never been investigated in intact exercising muscles and a robust model had not yet been developed, a limited number of mice were initially used to explore whether upregulation of RhoGDIα could be achieved. The rationale was that this exploration would direct future experimental work whereby the model could be refined. Indeed, although the sample size limits the statistical interpretation of the data, the observations of this study give impetus to test the RhoGDIα overexpressing mice under more intense exercising conditions or contractions or stretch, where at least Rac1 activation is known to be greater than what was achieved within the present study. While this study aimed to increase the actions of RhoGDI by overexpression, it would also be useful to supress RhoGDI either by genetic knockdown or by chemical blockade. This is particularly motivating given that RhoGDIα knockdown appears to increase insulin-stimulated glucose

uptake (Moller *et al.*, 2017), a stimulus which potently increases Rac1 activation. However, the challenge with any genetic manipulation is the potential for other signalling proteins to compensate. Chemical inhibitors in isolated muscle models are useful in circumventing such potential genetic compensatory effects, however, to our knowledge specific inhibitors of RhoGDIα are lacking. Future experimental work could also deliver the rAAV-6 via intramuscular injection (Muraine *et al.*, 2020) directly into superficial muscles without also targeted the liver and heart that occurs with systemic delivery (Blankinship *et al.*, 2004; Zincarelli *et al.*, 2008).

In conclusion, the results of this study demonstrate that overexpression of RhoGDIα did not reduce muscle glucose uptake during moderate-intensity treadmill running in mice. In contrast with the hypothesised role of RhoGDIα as a negative regulator of Rac1 signalling, the exercise-stimulated activation of the Rac1 downstream target PAK1 was not perturbed by RhoGDIα overexpression. However, it is possible that any inhibition of Rac1 by RhoGDIα was overcome by a compensatory increase in Rac1 protein content. Findings from this study encourage further work to unravel the complex regulation of RhoGDI-Rac1 in muscle models.

CHAPTER SIX: GENERAL DISCUSSION

6.1. Overview

This thesis investigated mechanisms regulating skeletal muscle glucose uptake during exercise using stretch, contraction, and *in vivo* exercise mouse models with a focus on nitric oxide synthase (NOS) and Rac1. A major finding, which contrasted to previous findings from our group, was that NOS inhibition did not attenuate *ex vivo* contraction-stimulated muscle glucose uptake in mice (Chapter Four). In addition, muscle stretching which stimulates glucose uptake, was not attenuated by NOS inhibitors or genetic deletion of eNOS or nNOSμ (Chapter Three). Further, I report that during stretch or contraction, NOS and Rac1 signalling are probably not linked since activation of Rac1, which increases skeletal muscle glucose uptake did not require NOS activation and vice versa (Chapter Three and Chapter Four). To investigate how Rac1 regulates skeletal muscle glucose uptake, the role of RhoGDI, a protein that has been suggested to negatively regulate Rac1 signalling in cell models, was examined. However, I found that RhoGDI overexpressing mice had similar Rac1 activation and skeletal muscle glucose uptake during *in vivo* exercise as control mice (Chapter Five). Taken together these findings in mice indicate that neither NOS or RhoGDI regulate Rac1 signalling or glucose uptake during contractions or exercise in mice. Importantly, findings of this thesis question the role of NOS in regulating skeletal muscle glucose uptake during contractions in mice.

6.2. Is NOS an important player in regulating muscle glucose uptake during contraction?

Human studies have largely supported a role for NO/NOS in regulating skeletal muscle glucose uptake during moderate-intensity exercise (Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Mortensen *et al.*, 2007). Rodent studies have attempted to unravel the underlying mechanisms involved, however, in contrast to human data, findings from rodent studies have been equivocal. Our group (Stephens *et al.*, 2004; Ross *et al.*, 2007; Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b) and others (Balon & Nadler, 1997) have previously shown that contraction-stimulated glucose uptake is attenuated by the NOS inhibitors L-NMMA or L-NAME in rodents. In contrast, several other studies (Etgen *et al.*, 1997; Higaki *et al.*, 2001; Inyard *et al.*, 2007), including one of our own (Hong *et al.*, 2015a), have reported no effect of NOS inhibition of skeletal muscle glucose uptake with contraction.

Experimental differences such as rodent strain examined, contraction intensity, and timing of glucose uptake measurement has been a prevailing argument as to why conflicting findings exist (McConell & Kingwell, 2006; Hong *et al.*, 2014). Therefore in this thesis, I employed an *ex vivo* contraction model that our lab has previously used to show that NOS regulates contraction-stimulated glucose uptake (Merry *et al.*, 2010b; Merry *et al.*, 2010c; Hong *et al.*, 2015b). However, unexpectedly, and in contrast to our previous results, I found a clear dissociation between NOS and contraction-stimulated glucose uptake in rodent muscle. Nonetheless, my results are in line with other preliminary work from our laboratory

using an *ex vivo* mouse contraction model (S Betteridge, A Betik, M Zhang, G McConell, *unpublished findings*). Likewise, unpublished work from Prof Erik Richter's group in Demark also found that L-NMMA did not inhibit contraction-stimulated glucose uptake in EDL mouse muscle *ex vivo* (L. Sylow, *personal communication*). Although these are surprising findings given our previous findings on the role of NO in skeletal muscle glucose uptake, this is not the first time that our group has indicated a disconnect between NOS activity and glucose uptake in mice. In previously published work, normal increases in glucose uptake were observed in mice lacking nNOSμ during contraction (Hong *et al.*, 2015b) and exercise (Hong *et al.*, 2016) despite there being no increase in NOS activity during the contraction/exercise. However, in one of those studies, L-NMMA attenuated *ex vivo* contraction-stimulated glucose uptake in nNOSμ KO mice despite NOS activity not increasing with contraction (Hong *et al.*, 2015b). It is worth postulating whether this reflects an off-target effect of L-NMMA given the lack of NOS activity in these mice. However, in that study (Hong *et al.*, 2015b) the highly compartmentalised nature of NOS signalling (Iwakiri *et al.*, 2006) was not able to be considered as the measurement of NOS activity was on whole muscle. It is possible that other NOS isoforms (eNOS or nNOSβ) were involved (Percival *et al.*, 2010) and activated in precise cellular locations and this was not detected using the NOS activity assay. Indeed, nNOSμ KO mouse muscle has been argued to express nNOSβ (Percival *et al.*, 2010), but this remains to be tested in the context of skeletal muscle glucose uptake.

Despite this, since blocking the increase in contraction-stimulated NOS activity did not attenuate skeletal muscle glucose uptake, findings from this thesis provide clear evidence that NOS is not essential in the regulation of skeletal muscle glucose uptake during contraction in mice. However, given that a number of studies support a role of NOS in regulating muscle glucose uptake, the involvement of NOS in this regard should not be discounted, but at the least, we should question the extent to which NOS plays a role. Nonetheless, the exact experimental conditions whereby NOS does indeed play a role in skeletal muscle glucose uptake in rodent models is not clear. We should, however, keep in mind that human studies on this topic have been more convincing (Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Mortensen *et al.*, 2007) and further research should focus on human trials to better understand how NOS regulates skeletal muscle glucose uptake during exercise.

6.3. NOS Does Not Interact with Rac1/PAK Signalling Pathway

Studies by Sylow and colleagues have placed Rac1 as a key signalling mediator of skeletal muscle glucose uptake during stretch, contraction, and exercise in rodent models (Sylow *et al.*, 2013b; Sylow *et al.*, 2015; Sylow *et al.*, 2016; Sylow *et al.*, 2017b). In this thesis, the motive to explore the potential link between Rac1 and NOS in regulating skeletal muscle glucose uptake was based on evidence from previously published studies reporting that increased NO levels in cultured cells resulted in an increase in Rac1 activity (Godfrey & Schwarte, 2010; Eller-Borges *et al.*, 2015). In addition, there is evidence that Rac1 is

susceptible to redox modifications by reactive nitrogen species (Heo & Campbell, 2005), raising the possibility that NO could directly interact with Rac1.

In Chapter Three, passive-stretch applied to isolated muscles *ex vivo* increased PAK signalling but did not increase NOS activation suggesting that Rac1/PAK signalling does not require NOS activation and vice versa. In Chapter Four, however, NO donor-stimulated glucose uptake was blocked by a Rac1 inhibitor suggesting that NO can increase Rac1 activity. This finding fits with a previous study in which Rac1 activity was increased in C2C12 cells when incubated with a NO donor (Godfrey & Schwarte, 2010). However, when tested during contraction (Chapter Four), Rac1/PAK signalling was not affected by NOS inhibition suggesting that NOS does not regulate Rac1 signalling in this setting. One explanation for the differences between the NO donor and contraction results is that NO donor treatment does not correspond to the same intracellular NO concentration to that of endogenously produced NO (nNOS and eNOS). Indeed, in one study, NO donors were reported to result in a diffuse spatial distribution of NO, whereas NOS-derived NO was primarily confined to targets within proximity of NOS enzymes (Iwakiri *et al.*, 2006). Therefore, while the NO donor experiments suggest that NO may be able to activate Rac1, this pathway may not be required under more physiological conditions of contraction.

The observation that Rac1 inhibition, but not NOS inhibition, attenuates contraction-stimulated skeletal muscle glucose uptake (Chapter Four) suggests that NOS is not involved in Rac1-mediated glucose uptake. While the data showing that NOS does not contribute to

glucose uptake would be considered a “negative” finding, it does however provide knowledge in refining signalling mediators involved in stretch- and contraction-activated pathways.

6.4. Rac1 signalling towards skeletal muscle glucose uptake

There is good evidence that Rac1 plays a major role in skeletal muscle glucose uptake during exercise and high-intensity muscle contractions (Sylow *et al.*, 2013b; Sylow *et al.*, 2017b), however, this is probably not the case at lower exercise intensities. Indeed, one study has shown that Rac1 activation was found to be greater at higher than lower exercise intensity levels (Sylow *et al.*, 2013b). In Chapter Five, Rac1 was not activated by treadmill running (60% max running speed) in gastrocnemius muscle and only tended to be activated in quadriceps muscle despite significant increases in glucose uptake. This suggests that at least in the gastrocnemius, Rac1 was not involved in the glucose uptake signal at the exercising intensity tested, which was slightly lower than other studies involving Rac1 KO mice (65% max running speed) (Sylow *et al.*, 2016; Sylow *et al.*, 2017b). Future studies attempting to examine mechanisms involving Rac1 signalling during exercise should therefore consider using higher exercising intensities.

The mechanisms acting upstream of Rac1 signalling in the context of skeletal muscle glucose uptake are not clear. A logical starting point in identifying the proteins involved has been to examine whether other known glucose uptake regulators act upstream of Rac1. Such studies have so far reported that AMPK (Sylow *et al.*, 2013b; Sylow *et al.*, 2017b), mTORC2

(Kleinert *et al.*, 2017), NOS (Chapter Three and Chapter Four) do not act as upstream regulators of Rac1 signalling during stretch, contractions, or exercise.

There is, however, promising evidence from cell culture studies suggesting that RhoGDI acts upstream of Rac1 to negatively regulate its signalling (DerMardirossian *et al.*, 2004; Moissoglu *et al.*, 2006; Bozza *et al.*, 2015). Therefore, in this thesis, the role of RhoGDI was examined in exercising mice (Chapter Five). In mice overexpressing RhoGDI, which should inhibit Rac1, exercise-stimulated Rac1 signalling and muscle glucose uptake was similar to control mice. This was surprising given that preliminary evidence in muscle cells reported that RhoGDI knockdown increased basal glucose uptake (Moller *et al.*, 2017), while in another study, increasing RhoGDI levels in KB cells was associated with reduced Rac1 signalling (Nishiyama *et al.*, 1994). Interestingly in this thesis, it was found that Rac1 protein levels were greater in RhoGDI overexpressing mice, which may have contributed to the maintenance of Rac1/PAK signalling. However, given that Rac1 only tended to be activated by treadmill running, the extent to which it contributed to the increase in glucose uptake in the present study is not clear. Future work is required to examine the role of RhoGDI during stretching (Sylow *et al.*, 2015), contractions (Sylow *et al.*, 2013b), or higher-intensity exercise (Sylow *et al.*, 2016; Sylow *et al.*, 2017b) where Rac1 activation would be expected to be more pronounced than what was achieved in the present study.

The increased RhoA protein, in line with the increase in Rac1, is an interesting point. RhoA has been reported to play a role in adiponectin-stimulated glucose uptake in primary

cardiomyocytes (Palanivel *et al.*, 2014), and in insulin-stimulated glucose uptake in adipocytes and L6 myoblasts (Furukawa *et al.*, 2005; Duong & Chun, 2019). However, the role of RhoA in regulating skeletal muscle glucose uptake has not been tested, and nor was it followed experimentally in this thesis. Future experimental work should be carried out to examine whether RhoA and Rac1 serve overlapping roles in regulating skeletal muscle glucose uptake during contractions or exercise.

6.5. Challenges, Limitations, And Perspectives

In this thesis, phosphorylation of the Rac1 downstream kinase PAK was used as a surrogate readout of Rac1 activation. Rac1 inhibition has been shown to completely block the increase of p-PAK1/2 during contractions and Rac1 KO mice have no increase in p-PAK1/2 during contractions (Sylow *et al.*, 2013b). This suggests that Rac1 activity is a requirement of PAK activation during contractions, and it is unlikely that other enzymes are causing the contraction or exercise induced increase in p-PAK1/2. It is acknowledged that direct measurement of Rac1 in its GTP-bound active form would have been a better assessment of Rac1 activity, however, this is difficult to conduct in mature muscle preparations (L. Sylow, *personal communication*). Indeed, in the lab where experiments for Chapter Five were carried out, Rac1-GTP levels are not reported in mature muscle models by that lab. More recently it has been suggested that that the role in PAK in contraction-stimulated glucose uptake is probably minor. This was based on one study showing that contraction-stimulated glucose uptake is normal in PAK1 KO mice and only slightly

attenuated in PAK2 KO mice (Møller *et al.*, 2020). Although at this point the role of PAK in contraction-stimulated glucose uptake is not clear, given the evidence that p-PAK mirrors Rac1 activity in contracting muscle (Sylow *et al.*, 2013b) its use as a readout of Rac1 activity is probably suitable.

It should be noted that both male and female mice were used in this thesis. Although the experimental design did not seek to determine potential sex differences in skeletal muscle signalling or glucose uptake, there is some published evidence that such differences may exist. Unfortunately, studies directly investigating potential sex differences in muscle glucose uptake during exercise are somewhat limited. Human studies have reported that glucose disposal is similar in males and females during moderate and intense exercise (Marliss *et al.*, 2000; Roepstorff *et al.*, 2002; Ruby *et al.*, 2002). One study using C57BL/6 mice reported that females have higher soleus and EDL muscle glucose uptake at rest and in the recovery period following prolonged swimming exercise (Kim *et al.*, 2006). Previous work from our group found no sex differences in skeletal muscle glucose uptake of nNOSμ knockout or wildtype littermates mice during contraction (Hong *et al.*, 2015b) or treadmill running (Hong *et al.*, 2016). During moderate-intensity exercise, human studies have reported both no difference (Vissing *et al.*, 2008), and higher levels (Roepstorff *et al.*, 2006) of AMPK activity in males compared to females. Any difference in AMPK signalling and effect on skeletal muscle uptake is unclear given the now questionable role of AMPK signalling during exercise (McConell, 2020).

In Chapter Three and Chapter Four, measurement of NO levels during stretch and contraction were estimated by the use of a radiolabelled NOS activity assay. It is important to note that this is an endpoint assay which provides an indirect measure of NO production under optimal *in vitro* conditions. This assay does not provide information on potential fluctuations of NO production or compartmentalized signalling (Iwakiri *et al.*, 2006) that real-time measurements in cells can confer. In Chapter Three, the finding that NOS activity did not increase with stretch may have therefore been due to the inability to detect initial bursts or important changes in NOS activation, or the spatial locations of NO during the stretching protocol. Measurement of NO bioavailability with fluorescent probes in a real-time manner under different muscle stretching and contracting regimens would be a useful strategy to clarify NO production in muscle in a more sensitive way than NOS activity measures. If we consider that under some conditions NOS does indeed act to regulate glucose uptake during contraction, it is important to determine the exact sources of NO and spatial signalling in mature rodent muscle models. That said, NOS inhibition did not affect glucose uptake in this scenario (rodent muscle), so even if NO bursts and spatial compartmentalisation are present, the effect of NO is negligible in these experiments.

Muscle glucose uptake during exercise has been proposed to involve separate signalling arms linked mechanical- and metabolic-mechanisms (Jensen *et al.*, 2014; Sylow *et al.*, 2015). Muscle stretching models have been used to delineate the proteins involved in the mechanical-signalling pathway. The rationale to explore stretch-stimulated glucose uptake in the context of NOS in Chapter Three was largely based on studies reporting that

NOS plays a role in contraction-stimulated glucose uptake and that stretching can stimulate NO release (Tidball *et al.*, 1998). In retrospect, it could be argued that results from the contraction study (Chapter Four), would indicate there be no need to explore NOS in stretching since stretch signals should be also activated by contraction. However, for this thesis, the stretch experiments were conducted prior to the contraction experiments and were done so under the premise that NOS was indeed involved in contraction-stimulated glucose uptake of rodent muscle, as our previous studies have described. Nonetheless, findings from Chapter Three contribute important empirical evidence into refining the signalling pathways involved in stretch and have since been published (Kerris *et al.*, 2019).

Rodent models involving transgenic modifications and pharmacological inhibitors to block targeted signalling mediators, and isolation of specific physiological systems through *ex vivo* and *in situ* models provide an important tool in biomedical research. However, findings from this thesis, as well as the current body of literature of NOS studies highlight that sufficiently robust rodent models are currently lacking in this area of research. Overall, some models support, while others refute a role for NOS in glucose uptake during contractions and it is unclear as to exactly under which experimental conditions that NOS regulates contraction-stimulated glucose uptake. Nonetheless, the importance of published NOS human studies should be kept in mind, and in particular should be a focus of future research efforts. Importantly, an earlier human trial from our group suggested that people with type 2 diabetes have a greater reliance on NOS to achieve muscle glucose uptake during exercise compared with healthy individuals (Kingwell *et al.*, 2002). There is still much to

clarify however since previous NOS inhibitor glucose uptake studies in humans (Bradley *et al.*, 1999; Kingwell *et al.*, 2002) did not collect muscle biopsy specimens and therefore lack information on protein signalling with NOS inhibition. Furthermore, it needs to be determined whether the effects of NOS inhibition on glucose uptake in exercising humans is due solely to intracellular muscle factors, or potential NOS inhibition effects on microvascular blood flow. Our group showed that NOS inhibition attenuated the increase in skeletal muscle glucose uptake during rat *in situ* contractions independently of microvascular blood flow (Ross *et al.*, 2007) but no study has examined this in humans.

6.6. Conclusions

In summary, the major conclusions of this thesis are:

1. NO/NOS does not play a role in stretch-stimulated glucose uptake in isolated mouse muscle
2. Rac1/PAK1 signalling with stretch and contraction does not require NOS activation in mouse muscle.
3. While exogenous NO stimulates mouse muscle glucose uptake presumably via Rac1, NOS-derived NO does not appear to play a role in contraction-stimulated mouse skeletal muscle glucose uptake and is not linked with Rac1 signalling.
4. The finding that NOS inhibition did not attenuate the increase in contraction-stimulated glucose uptake in isolated mouse skeletal muscle is in contrast with

previous work from our group and others. Reasons for the conflicting findings remain unclear but nonetheless question the hypothesis that NOS plays an essential role in regulating skeletal muscle glucose uptake during contraction in mice.

5. Upregulation of RhoGDI in muscle, which should inhibit Rac1, does not appear to attenuate muscle glucose uptake during exercise *in vivo*. This is possibly because Rac1 protein content was also upregulated by RhoGDI upregulation which may have maintained Rac1/PAK1 signalling. Future work is required to examine Rac1/PAK1 signalling mechanism under conditions where Rac1 is activated to a greater extent such as during stretch, contractions, or higher-intensity exercise.

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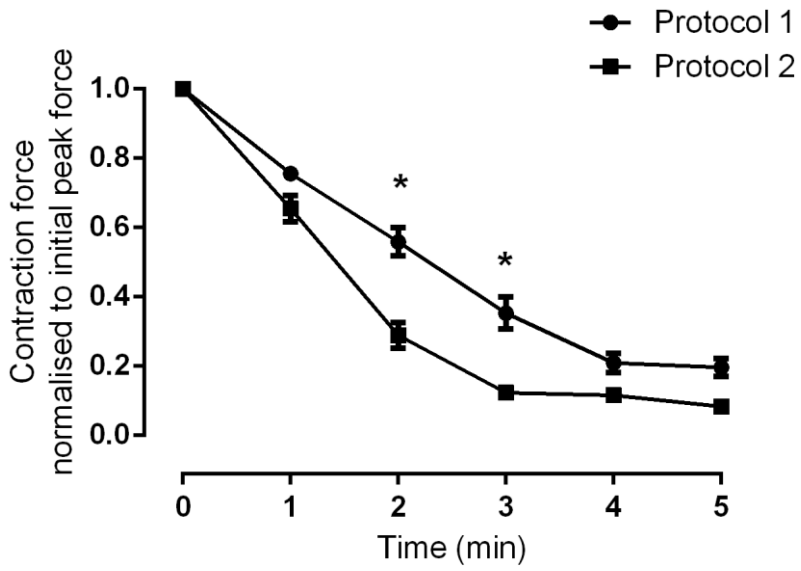
APPENDIX A: CONTRACTION PROTOCOL FORCES

Pilot experiments showing contraction force output (normalised to initial peak force) of isolated EDL muscles stimulated to contract during first 5 minutes of 10 minutes stimulation protocol, comparing Protocol 1 (0.2 ms pulses, 60 Hz, 12 volts, 350 ms train duration, at 12 contractions per minute) and Protocol 2 (0.2 ms pulses, 100 Hz, 30 volts, 2000 ms train duration at 4 contractions per minute). N = 10 and 7 respectively. * $P < 0.001$.



APPENDIX B: WESTERN BLOT PROTEIN LOADING

Representative coomassie stained blots for A) Soleus, B), Quadriceps, and C) Gastrocnemius muscles for experiments reported in Chapter Five.