Exercise intensity and glycaemic control: The role of redox status and redox-sensitive protein kinase signalling in humans.

This thesis is submitted in fulfilment of the requirements for the degree

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by

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ABSTRACT

Physical inactivity and obesity are associated with elevated systemic oxidative stress and the activation of JNK, p38 MAPK, NF-κB, and protein kinase C (PKC) signalling pathways in skeletal muscle. Sustained activation of these stress and mitogen activated protein kinase (SAPK) pathways are associated with impaired glycaemic control, and the development and progression of cardiometabolic disease. Paradoxically, acute exercise also increases oxidative stress and SAPK signalling, yet glycaemic control and skeletal muscle function are enhanced. Research now supports a role for the transient induction of oxidative stress and associated activation of SAPK signalling in the physiological response and adaptation to acute exercise and exercise training. High-intensity interval exercise (HIIE) is a potent exercise stimulus for the improvement of metabolic health and skeletal muscle adaption, however the effect of HIIE on oxidative stress and SAPK signalling is unclear. The aims of this thesis were to explore the effect of HIIE on glycaemic control, exercise-induced oxidative stress, and skeletal muscle SAPK signalling, in a series of independent but related studies.

Firstly, this thesis reports the novel finding that a single session of sprint interval exercise (SIE: 4 x 30 second all out sprints, 4.5 minutes recovery periods), which consisted of substantially less total work than HIIE (5 x 4 minutes at 75% W_{max}, 2-minute recovery periods) and CMIE (30 minutes at 50% W_{max}), elicited greater post-exercise plasma catalase activity and skeletal muscle NF-κB protein phosphorylation. Furthermore, SIE elicited a similar increase in skeletal muscle p38 MAPK and JNK phosphorylation, and a similar decrease in skeletal muscle PKC δ/θ phosphorylation. These findings suggest that the superior skeletal muscle adaptation frequently reported after SIE may occur through NF-κB protein signalling, whereas p38 MAPK and JNK protein signalling may play a lesser role. Surprisingly, only CMIE and HIIE elicited a decrease in phosphorylation of the downstream glucose uptake signalling protein AS160 three hours after exercise, despite lower AS160 phosphorylation immediately after SIE. These findings indicate that post-exercise AS160 phosphorylation, an important regulator of contraction and insulin-stimulated glucose uptake, is influenced in an exercise-intensity and post-exercise time-course dependent manner.

The major finding of the second study was that whole-body insulin stimulation in obese middle-aged males, via hyperinsulinaemic-euglycaemic clamp, increases skeletal muscle oxidative stress, JNK, p38 MAPK, and NF-κB protein phosphorylation, while increasing plasma antioxidant activity and decreasing plasma oxidative stress. These findings support the role of redox homeostasis and SAPK signalling in human insulin stimulated glucose uptake. Importantly, insulin stimulated phosphorylation of JNK, p38 MAPK and NF-κB signalling were augmented by a prior session of HIIE (4 x 4 minutes at 95% HR_{peak}, 2-minute recovery periods), whereas PKC δ/θ phosphorylation decreased. These findings provide new evidence that exercise-induced transient activation of SAPK signalling does not appear to impair insulin protein signalling or insulin sensitivity. On the contrary, evidence provided indicates that activation of SAPK signalling pathways may contribute to the post-exercise enhancement of insulin signalling and whole-body insulin sensitivity in humans.

Excess postprandial hyperglycaemia and oxidative stress contribute to the development and progression of cardiometabolic disease. The final study provides evidence that low-volume HIIE (LV-HIIE: 8 x 1 minutes at 100% W_{max}, 1 min recovery periods) in overweight inactive adults performed 1 hour after consumption of a standard breakfast increases post-prandial oxidative stress compared to continuous moderate-intensity exercise (CMIE: 38 minutes at 50% W_{max}), despite similar decreases in postprandial glucose. In contrast, post-prandial oxidative stress was attenuated 24 hours after exercise to a greater extent with LV-HIIE. LV-HIIE and CMIE both improved 24-hour post-exercise glycaemic control, as measured by continuous glucose monitoring, to a similar extent. These findings indicate that a single session of LV-HIIE has lasting benefits on glycaemic control and postprandial oxidative stress in overweight adults, despite consisting of substantially less total work and time-commitment than traditional CMIE.

This thesis provides evidence that SIE and HIIE are effective exercise modes for the activation of skeletal muscle SAPK signalling and enhancement of glycaemic control in healthy and obese populations. Furthermore, findings presented within this thesis support a beneficial role of exercise-induced alterations in redox status and the activation of skeletal muscle SAPK signalling in human glycaemic control.

STUDENT DECLARATION

“I, Lewan Parker, declare that the PhD thesis entitled “Exercise-intensity and glycaemic control: The role of redox status and redox-sensitive protein kinase signalling in humans” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, and references. 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 8/9/2016

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•OH: hydroxyl radical

4-HNE: 4-hydroxynonenal

ADP: adenosine diphosphate

AGE: advanced glycation end product

Akt: protein kinase B

AMP: adenosine monophosphate

AMPK: 5' adenosine monophosphate-activated protein kinase

AOPP: advanced oxidation protein products

AS160: Akt substrate of 160 kDa

ATP: adenosine triphosphate

BAP: biological antioxidant potential

CaMK: ca2+/calmodulin-dependent protein kinase

CAT: catalase

CMIE: continuous moderate-intensity interval exercise

Cu-ZuSOD: copper-zinc superoxide dismutase

DNA: deoxyribonucleic acid

dROM: reactive oxygen metabolites

Ec-SD: extracellular superoxide dismutase

ETC: electron transport chain

G6P: glucose-6-phosphate

GLP-1: glucagon-like peptide-1

GLUT4: glucose transporter type 4

GPx: glutathione peroxidase

GSH: glutathione

GSK3: glycogen synthase kinase 3

H_2O_2: hydrogen peroxide

HbA1c: glycated haemoglobin

HHR: heart rate reserve

HIIE: high-intensity interval exercise

HK: hexokinase

IL-6: interleukin 6

IRS-1/2: insulin receptor substrate 1 and 2

IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

JNK: c-Jun N-terminal kinases

LOOH: lipid hydroperoxides

LV-HIIE: low-volume high-intensity interval exercise

MAPK: mitogen activated protein kinase

MDA: malondialdehyde

MKP: mitogen activated protein kinase phosphatases

Mn-SOD: Manganese superoxide dismutase

mTFA: mitochondrial transcription factor A

mTORC2: mechanistic target of rapamycin complex 2

mtTFA: mitochondrial transcription factor A

NADPH: nicotinamide adenine dinucleotide phosphate

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NO: nitric oxide

NOS: nitric oxide synthase

NOX: nicotinamide adenine dinucleotide phosphate-oxidase

NRF: nuclear respiratory factor

$O_2^{\cdot-}$: superoxide

$ONOO^{\cdot-}$: peroxynitrite

P38 MAPK: p38 mitogen-activated protein kinase

PDK1: phosphoinositide-dependent kinase-1

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K: phosphatidylinositol-3 kinase

PIP3: phosphatidylinositol (3,4,5)-trisphosphate

PKC: protein kinase C

PLA_2: phospholipases A2

PP2A: protein phosphatase 2

PTEN: phosphatase and tensin homolog

PTP: protein tyrosine phosphatase

PTP1B: protein-tyrosine phosphatase 1B

PUFA: Polyunsaturated fatty acids

RAC1: ras-related C3 botulinum toxin substrate 1

RNA: ribonucleic acid

ROS: reactive oxygen species

SAPK: stress and mitogen activated protein kinase

SIE: sprint interval exercise

SOD: superoxide dismutase

T2DM: type 2 diabetes mellitus

TBARS: thiobarbituric acid reactive substances

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Papers resulting directly (or indirectly) from this thesis which have been accepted/published or are under review:

Invited Literature Review:

1. **Parker, L.,** Shaw, C., Stepto, N.*, & Levinger, I.* (2017). Exercise and Glycemic Control: Focus on Redox Homeostasis and Redox-Sensitive Protein Signaling. Frontiers in Endocrinology, 8(87).

Original Research Articles:

2. **Parker, L.,** Trewin, A., Levinger, I., Shaw, C. S., & Stepto, N. K. (2017). The effect of exercise-intensity on skeletal muscle stress kinase and insulin protein signaling. PLoS ONE, 12(2), e0171613.
3. **Parker, L.,** Stepto, N.K., Shaw, C.S., Serpiello, F.R., Anderson, M., Hare, D.L., and Levinger, I. (2016). Acute high-intensity interval exercise-induced redox signaling is associated with enhanced insulin sensitivity in obese middle-aged men. Front. Physiol. 7, 411.
4. **Parker, L.,** Shaw, C.S., Banting, L., Levinger, I., Hill, K.M., Mcainch, A.J., and Stepto, N.K. (2016). Acute low-volume high-intensity interval exercise and continuous moderate-intensity exercise elicit a similar improvement in 24-h glycemic control in overweight and obese adults. Front. Physiol. 7, 661.**2.**
5. Levinger, I., Jerums, G., Stepto, N.K., **Parker, L.**, Serpiello, F.R., Mcconell, G.K., Anderson, M., Hare, D.L., Byrnes, E., Ebeling, P.R., and Seeman, E. (2014). The effect of acute exercise on undercarboxylated osteocalcin and insulin sensitivity in obese men. J. Bone Miner. Res. 29, 2571-2576.
6. **Parker, L.,** Trewin, A., Levinger, I., Shaw, C. S., & Stepto, N. K. (2017). Exercise-intensity dependent alterations in plasma redox status do not reflect skeletal muscle redox-sensitive protein signaling. JSAMS.
7. **Parker, L.**, Mcguckin, T.A., and Leicht, A.S. (2014). Influence of exercise intensity on systemic oxidative stress and antioxidant capacity. Clin. Physiol. Funct. Imaging 34, 377-383.

(Note: This publication was not based on data obtained from this thesis. However, data analysis and interpretation, and manuscript preparation was conducted during the PhD candidature.)

Conference papers resulting directly from this thesis:

Oral: **Parker, L.**, Shaw, C., Banting, L., Hill, K., McAinch, A., Levinger, I., Stepto, N. (2016). Low-volume high-intensity interval exercise improves glycaemic control similarly to continuous moderate-intensity exercise despite increased postprandial oxidative stress. European College of Sport Science (Vienna, Austria).

Oral: **Parker, L.**, Stepto, N., Shaw, C., Serpiello, F., Anderson, M., Hare, D., Levinger, I. (2016). Exercise-induced oxidative stress and insulin sensitivity in obese middle-aged men: Investigation of a redox paradox. Exercise and Sport Science Australia (Melbourne, Australia)

Oral: **Parker, L.**, Stepto, N., Shaw, C., Serpiello, F., Anderson, M., Hare, D., Levinger, I. (2015). Acute high-intensity exercise-induced redox signalling is associated with enhanced insulin sensitivity in obese middle-aged men. Australian Physiological Society (Hobart, Australia)

Oral: **Parker, L.**, Shaw, C., Banting, L., Hill, K., McAinch, A., Levinger, I., Stepto, N. (2015). Time-efficient high-intensity exercise improves glycaemic control similarly to moderate-intensity exercise in overweight humans. Sports Medicine Australia (Gold Coast, Australia).

Oral: **Parker, L.**, Shaw, C., Banting, L., Hill, K., McAinch, A., Levinger, I., Stepto, N. (2015). Time-efficient high-intensity exercise improves glycaemic control similarly to moderate-intensity exercise in overweight humans. ISEAL Higher Degree Research Conference (Melbourne, Australia).

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Oral: **Parker, L.**, Stepto, N., Shaw, C., Serpiello, F., Anderson, M., Hare, D., Levinger, I. (2014)**.** The effects of acute exercise on redox homeostasis and insulin sensitivity in obese men. ISEAL Higher Degree Research Conference (Melbourne, Australia).

PREFACE

Data collection, analysis, and interpretation presented in this thesis is my own. Significant contributions include:

- In Chapter 3, Adam Trewin designed the study and assisted with participant recruitment; Dr. Andrew Garnham performed muscle biopsies; and Danielle Hiam, Christopher Shaw, and Itamar Levinger assisted with data collection.
- In Chapter 4, Itamar Levinger and David Hare designed the study and recruited participants; Itamar Levinger and Nigel Stepto assisted with the conductance of the hyperinsulinemic euglycemic clamp; Dr. Andrew Garnham and Dr. Mitchell Anderson performed muscle biopsies; and Itamar Levinger, Fabio Serpiello and Nigel Stepto assisted with data collection.
- In Chapter 5, Nigel Stepto, Andrew McAinch, and Christopher Shaw assisted with the design of the study; Nigel Stepto, Christopher Shaw and Lauren Banting assisted with participant recruitment; and Lauren Banting and Christopher Shaw assisted with data collection.

CHAPTER 1. INTRODUCTION.

More than 1.4 billion adults worldwide are overweight or obese with this number increasing every year (471). Excess adipose tissue is associated with the development of insulin resistance and subsequent development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (206, 364). In 2010 over 285 million adults worldwide were estimated to have diabetes (390). In Australia, over 1 million individuals have been diagnosed with T2DM and it is estimated that a further 1 million people are undiagnosed (389). Obesity and T2DM place a significant social and economic burden on society with recent estimates suggesting a global annual cost of over 376 billion USD (486) and 60 billion AUD in Australia (389). Although physical activity is an effective means for the prevention and management of metabolic disease (334, 364, 455, 483), approximately 60% of adults are considered physically inactive (169, 405).

Living organisms are constantly undergoing oxidation-reduction (redox) reactions in order to maintain a state of redox homeostasis. Physical inactivity and obesity can lead to a redox state known as oxidative stress. Oxidative stress occurs when reactive oxygen species (ROS) overwhelm antioxidant defences, shifting the redox environment towards one conducive to oxidative modification and/or damage to proteins, lipids and deoxyribonucleic acid (DNA) (119, 122). Chronic oxidative stress is associated with over 100 pathologies including insulin resistance and T2DM (436), mediated in part through the sustained activation of redox-sensitive stress and mitogen activated protein kinases (SAPK) (421). Paradoxically, a single session of exercise also increases ROS, oxidative stress and SAPK signalling, albeit transiently (120, 228), yet exercise is known to alleviate many of the complications associated with chronic oxidative stress induced pathologies (483). A growing body of evidence now supports exercise-induced ROS and SAPK signalling as a necessary requirement and beneficial modulator of metabolic health (155, 228, 332, 346). It appears that higher-intensity exercise elicits greater oxidative stress and antioxidant activity (161, 237, 323, 380). Less clear, however, is the influence of exercise-intensity on SAPK signalling, and whether increased

exercise-induced oxidative stress and/or SAPK signalling with higher-intensity exercise plays a role in improved metabolic health.

High-intensity interval exercise (HIIE) is a trending exercise mode that consists of short bursts of high-intensity exercise, interspersed with active or passive recovery periods (418). HIIE is reported to promote similar, and in some cases greater enhancements in skeletal muscle adaptation and glycaemic control when compared to more traditional continuous moderate-intensity exercise (CMIE) modes (141, 348, 460). However, the mechanisms for increased skeletal muscle adaptation and improved glycaemic control are unclear. The transient induction of ROS, oxidative stress, and/or skeletal muscle SAPK signalling after endurance exercise may play a role in the acute post-exercise enhancement of insulin sensitivity (253), however the effects of HIIE are unclear. As such, the current thesis aimed to explore the apparent redox paradox by investigating the effect of HIIE on post-exercise redox status, skeletal muscle redox-sensitive SAPK signalling, and glycaemic control.

CHAPTER 2. REVIEW OF LITERATURE.

This chapter informed an invited literature review published in Frontiers in Endocrinology, Diabetes.

Parker, L., Shaw, C., Stepto, N.*, & Levinger, I.* (2017). Exercise and Glycemic Control: Focus on Redox Homeostasis and Redox-Sensitive Protein Signaling. Frontiers in Endocrinology, 8(87).

2.0 Redox biology.

2.0.1 Reactive oxygen species.

Free radicals consist of any chemical species containing one or more unpaired electrons in the outer orbital shell, and as such are typically unstable and highly reactive. Although non-radicals typically contain paired electrons in their outer orbital shell (171), they are nevertheless chemically reactive and can interact with other molecules to form free radicals (171). Reactive oxygen species (ROS) is the collective term that refers to both free radicals and non-radical oxygen (O_2) generated derivatives (17, 73). ROS production in a biological system occurs through numerous sources including radiation, environmental pollutants, chemotherapeutics, psychological stress (291), normal and abnormal cellular substrate metabolism (122, 473), and mechanical and physiological stress induced through exercise (120, 122). ROS considered to be of biological importance consist of the free-radicals hydroxyl, superoxide (O_2^-), nitric oxide (NO), peroxyl radicals; and the non-radicals peroxynitrite, hypochlorous acid, hydrogen peroxide (H_2O_2), singlet oxygen, and ozone (17). It should be noted that reactive nitrogen species (85, 325) and reactive sulphur species (73, 143) also constitute separate radical groups with independent biological functions, however the focus of this thesis pertains primarily to that of ROS and as such discussion of the other radicals lies beyond the scope of the thesis.

2.0.2 Antioxidant defence.

Uncontrolled ROS production leads to exponential apoptotic and necrotic cellular death (366). Organisms have therefore evolved to encompass a complex system of ROS defence designed to maintain oxidation and reduction (redox) homeostasis through preventative mechanisms, repair mechanisms, physical defences, and antioxidants (436). Intracellular and extracellular reducing agents possess the ability to neutralize ROS and/or inhibit its production, as such they are commonly referred to as antioxidants. Antioxidants include both endogenous (synthesized *in vivo*) and exogenous sources, and can be divided into enzymatic and non-enzymatic groups (436). Non-enzymatic antioxidants include reduced glutathione, uric acid, lipoic acid, bilirubin, coenzyme Q10, vitamin C, vitamin E, and carotenoids. Non-enzymatic antioxidants mitigate oxidative stress primarily

through the reduction of ROS and/or ROS intermediates, and termination of ROS mediated chain reactions (56, 57, 108, 317). Mitigation of ROS can also occur through indirect methods such as the synergistic activation of glutathione (GSH) resynthesis of vitamin C and E (384), regeneration of reduced vitamin E by vitamin C, GSH and lipoic acid (309, 340), and through coenzyme replenishment of enzymatic antioxidant defences such as with GSH and glutathione peroxidase (GPx) and glutathione S-transferase (269, 340).

The primary enzymatic antioxidant sources include superoxide dismutase (SOD), catalase (CAT), and the GPx system (153). SOD acts as a catalyst in the dismutation of O_2^- to form oxygen and the less reactive H_2O_2 (118). SOD therefore typically appears near sites of active O_2^- (428). The three isoforms of SOD include extracellular SOD (Ec-SOD) (264); manganese SOD (Mn-SOD) primarily found within the mitochondria matrix; and copper and zinc SOD (Cu-Zn-SOD) primarily found within the cellular cytosol and intermembrane mitochondrial space (458). Catalase is primarily located in peroxisomes and acts to reduce H_2O_2 to H_2O. However, the enzyme of GPx, which is mostly located in the mitochondria and cytosol, is preferentially recruited for the neutralization of H_2O_2 (12, 274). The neutralization of H_2O_2 via GPx results in the oxidation of GSH. Oxidized GSH is later converted back to its reduced form with the assistance of glutathione reductase, a biological process dependent upon sufficient nicotinamide adenine dinucleotide phosphate (NADPH) availability (42).

Exogenous sources of antioxidants include many of the previously mentioned non-enzymatic antioxidants such as Vitamin C and Vitamin E, but also include reducing compounds that directly scavenge and/or attenuate ROS production such as N-acetylcysteine and quercetin (18, 485).

2.0.3 Oxidative Stress.

Living organisms are constantly undergoing redox reactions to maintain a state of redox homeostasis (274). Under certain conditions ROS may overwhelm endogenous and/or exogenous antioxidant defences, promoting a redox imbalance in favour of oxidative stress. Oxidative stress is associated with both beneficial and harmful effects on health, and as such is defined as a redox imbalance that results in increased levels of ROS production and/or oxidant

biomarkers (310). During periods of oxidative stress, elevated ROS elicits oxidative damage and/or modification to lipids, proteins, ribonucleic acid (RNA), and DNA (436).

ROS are capable of direct and/or indirect oxidative modification to proteins (240). Sustained oxidation of proteins can result in disruptions in the normal functioning of the proteome including protein inactivation (97), modification of the protein side chains, fragmentation of peptide bonds (68), and structural unfolding and conformational changes (94). Likewise, ROS are implicated in oxidative damage to DNA, a process which ultimately results in strand breakage, DNA-protein cross-links and base alterations, and defective DNA transcription and translation leading to the synthesis of less protein and/or defective protein (84, 225, 357). In addition to DNA, both messenger and ribosomal RNA are vulnerable to oxidative damage which can lead to the disturbance of translational process' and impairment of protein synthesis (225). ROS induced damage to mRNA occurs primarily through the formation of highly reactive free radicals such as the hydroxyl radical (27) and appears to be selective and independent of the abundance of the mRNA species (225). Although RNA is highly susceptible to oxidative damage, considerably more so than DNA, protein and lipids (225), to the candidate's knowledge research has yet to investigate the effect of exercise-induced ROS production on RNA damage and the subsequent effects on protein synthesis and exercise adaption. However, considering that oxidative DNA damage is reported to occur following extreme muscle-damaging exercise regimes where free radical production is likely to be high (14, 339), damage to RNA and subsequent impairment of protein synthesis may also occur under similar oxidative conditions.

Lipids, especially polyunsaturated fatty acids, are susceptible to oxidative degradation (173), a process referred to as lipid peroxidation (57). Lipid peroxidation results in the formation of peroxyl radicals and hydroperoxides (57). Excess lipid peroxidation is associated with numerous diseased states in part through the production of secondary products such as malondialdehyde (MDA), propanal, hexanal, and the highly toxic 4-hydroxynonenal (4-HNE) (28, 336, 337, 377).

Chronic elevation of systemic oxidative stress is suggested to play a primary and/or secondary role in over 100 pathological conditions (436). These include accelerated ageing (319), cardiovascular disorders (97), cancerous malformations (468), rheumatoid arthritis, neurodegenerative conditions (215), decreased skeletal muscle force production (341, 351) and fatigue (118, 352), and insulin resistance, T2DM, and diabetic complications (122, 436). Considerable research has reported chronically elevated systemic oxidative stress and attenuated antioxidant defence in populations with chronic disease (119, 193, 218, 226, 265, 306). Elevated biomarkers of plasma oxidative stress correlate with classical cardio-metabolic risk factors such as increased circulating high-sensitivity C-reactive protein, greater waist to hip ratio, serum total cholesterol, serum triglycerides, and fasting blood glucose (21, 133, 208, 218, 226, 306). As such, the measurement of basal systemic oxidative stress has been proposed as a marker for predicting the onset of a disease, assessing the progression of a disease, and for evaluating the effect of interventions designed to treat oxidative stress associated disease (261).

2.1 Exercise-induced oxidative stress.

Acute exercise elicits a transient state of elevated ROS and may result in oxidative stress (120, 228). In contrast to chronic oxidative stress, the transient increase in ROS and oxidative stress elicited by exercise is reported to be beneficial and a necessary requirement for cellular function and adaptation to physiological stress (346).

2.1.1 Mechanisms for exercise-induced oxidative stress.

The direct measurement of ROS in humans is complex and expensive (42), as such, literature pertaining to the mechanisms of exercise-induced ROS production is scarce and almost non-existent in humans. Nevertheless, the primary mechanisms of intracellular and extracellular ROS generation in skeletal muscle are suggested to occur through NADPH oxidase (112, 370), xanthine oxidase (345), nitric oxide synthase (403), arachidonic acid release from cell membranes by phospholipase A2 (159, 307, 487), and mitochondrial oxidative phosphorylation (367) (Figure 2.1).

The degradation of adenosine triphosphate (ATP) during exercise results in the accumulation of adenosine diphosphate (ADP) and adenosine monophosphate (AMP) which is subjected to further degradation in the presence of inosine and adenosine leading to the formation of hypoxanthine (345). Concomitantly, the decrease in ATP availability contributes to a cascade of reactions which influence the ion channel regulation of cells leading to activation of xanthine oxidase (345). Hypoxanthine is catalysed by xanthine oxidase to form xanthine and uric acid, a process which produces O_2^- and hydroxyl radicals (70, 177, 315, 345, 481).

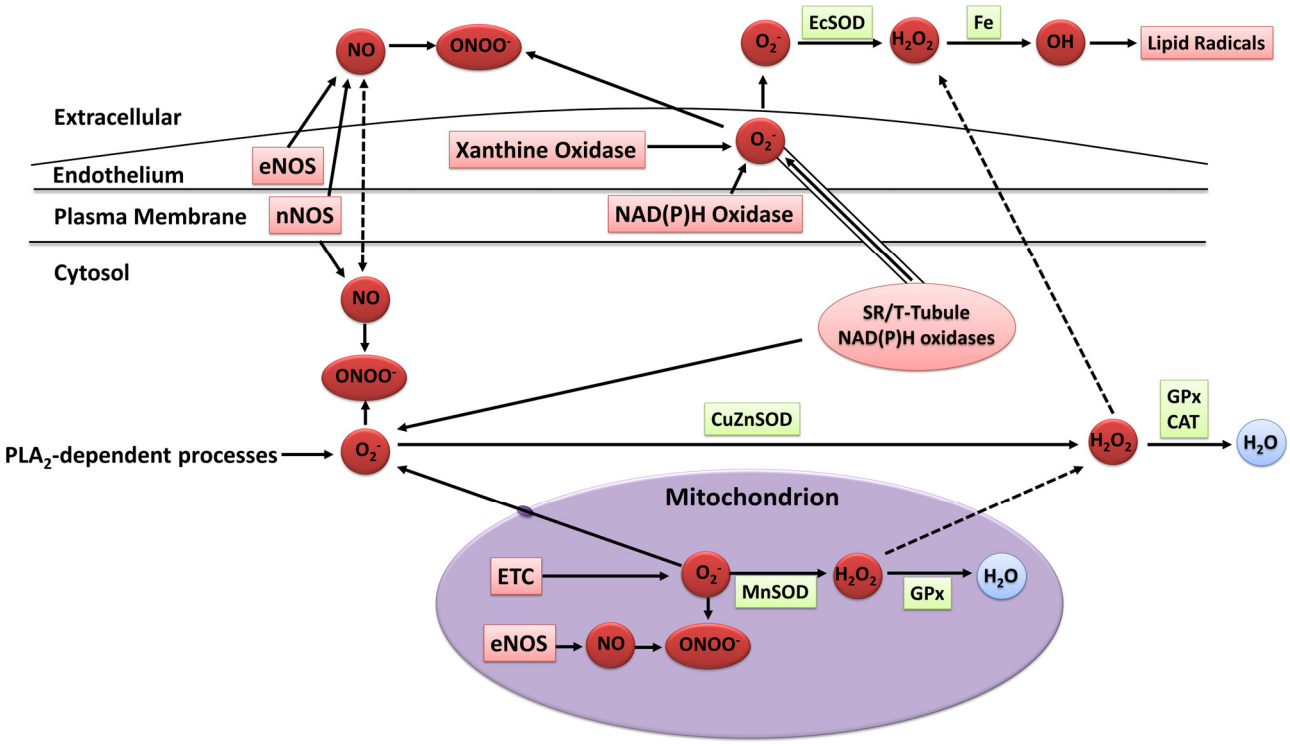

Figure 2.1: Sources of ROS in skeletal muscle. **ETC:** electron transport chain. **eNOS**: endothelial nitric oxide synthase. **nNOS:** neuronal nitric oxide synthase. **NO:** nitric oxide. **$ONOO^-$:** peroxynitrite. **OH:** hydroxyl radical. **O_2^-:** superoxide. **H_2O_2:** hydrogen peroxide. **H_2O:** Water. **ecSOD:** extracellular superoxide dismutase. **MnSOD:** manganese superoxide dismutase. **CuZuSOD:** Copper-zinc superoxide dismutase. **GPx:** glutathione peroxidase. **CAT:** catalase. **PLA_2:** phospholipase A2. **Fe:** iron. Adapted from Powers and Jackson (341), Powers et al. (342).

Skeletal muscle NADPH oxidase is found in the plasma membrane, sarcoplasmic reticulum, and transverse tubules, and is activated through membrane depolarization and/or after electrical stimulation of rat skeletal muscle and uses NADPH as a substrate to produce superoxide (112). Mitochondrial electron leak to molecular oxygen, and subsequent superoxide production, may also produce ROS during exercise (367). However, due to increased mitochondrial ATP

resynthesis and subsequent attenuation of the mitochondrial membrane potential, mitochondrial electron leak is now suggested to contribute only marginally to ROS production during muscular contraction (402, 439). Other potential mechanisms that result in elevated skeletal muscle and/or plasma oxidative stress include the oxidation of catecholamine's (97, 394), lactate accumulation (254, 290), elevated core body temperature (273), haemoglobin and myoglobin mediated auto-oxidation (202, 392, 449), and through post-exercise inflammatory and phagocytic responses including ischemic reperfusion, cytokine secretion, and respiratory burst (85, 126, 227, 328).

2.1.2 Measuring exercise-induced oxidative stress.

The gold standard for measuring ROS in human tissue is through the use of spin trapping via electron spin resonance (ESR) or electron paramagnetic resonance (24). However, this method is invasive, requires considerable technical experience, and is subject to artifactual generation of ROS during tissue preparation (201). Combined with the added complexities and costs involved with ESR, more practical and cost-effective methods that measure ROS induced oxidative damage by-products and derivatives are preferentially used in human research. The most common indirect measures of ROS include lipid peroxidation by-products such as conjugated dienes, ethane, pentane, lipid hydroperoxides, malondialdehyde, F_2-isoprostanes and 4-hydroxynonenal (4-HNE) (73, 343). Oxidative damage to DNA is often measured through 8-hydroxy-2'-deoxyguanosine (212, 213). Common biomarkers of oxidative damage and/or oxidative modification to protein include protein carbonyls and 4-HNE (42, 336).

Although plasma oxidative stress is commonly measured as an indicator of exercise-induced oxidative stress, the exact sources of systemic oxidative stress following skeletal muscle contraction are not well understood. Nevertheless, due to the large proportion of body mass that is constituted by skeletal muscle, it is proposed that skeletal muscle fibres, vascular cells, endothelial cells and/or blood cells residing within skeletal tissue are the main contributors of both the exercise-induced local and systemic oxidative stress response (197). Ex vivo skeletal muscle contraction studies have established the capacity of skeletal muscle to elicit systemic oxidative stress (77, 197, 313). The specific cell types that contribute to skeletal muscle ROS production likely include vascular smooth

muscle cells, endothelial cells, fibroblasts, erythrocytes and white blood cells, with skeletal muscle fibres suggested to play the biggest role in the generation of extracellular ROS during and after exercise (197, 326, 393). Other tissues such as the heart, liver, and lungs, may also contribute to the systemic increase in oxidative stress following acute exercise, but likely to a lesser degree (197).

Antioxidant defences transiently increase to mitigate increasing levels of ROS and oxidative stress to maintain redox homeostasis (155, 436). The measurement of antioxidant defence is therefore often used as a surrogate marker for increased ROS and/or oxidative damage in a biological system (436). The most common measures of antioxidant defence include antioxidant enzymatic activity of SOD, CAT, and GPx (461); the ratio of oxidized and reduced glutathione (318); and the measurement of overall antioxidant capacity through specific assays such as the total antioxidant capacity/status, trolox equivalent antioxidant capacity, ferric reducing ability of plasma, and biological antioxidant potential (33, 60, 323).

We previously explored the effects of acute exercise on systemic redox status through a commercially available automated free radical analytical system (FRAS4). The FRAS4 system can be used to analyse both the Biological Antioxidant Potential (BAP) and Reactive Oxygen Metabolite (dROM) concentration of human plasma. The BAP assay measures the plasma's ability to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions using a chromogenic substrate that changes colour intensity during this oxidation-reduction reaction. The sample can then be photometrically measured (absorption at 505 nm wavelength) using the FRAS4 system with values expressed in µmol F^{e2+}/L. Values of ≥ 2200 µmol F^{e2+}/L reflect normal levels of global antioxidant defence (431). The dROM assay quantifies total plasma hydroperoxides which constitute oxidative derivatives of phospholipids (and lipids), proteins and DNA. This test is based on the ability of transition metals to catalyse, in the presence of peroxides, the formation of free radicals which react with the chromogen N,N-diethyl-p-phenylenediamine to produce a coloured complex that can be photometrically quantified (absorption at 505 nm wavelength). Values are expressed in U CARR (Carratelli units) with values greater than 300 U CARR indicating different degrees of oxidative stress (431).

One of the most commonly adopted measures for systemic oxidative stress in biological samples is the TBARS assay. This assay measures the lipid peroxidation product malondialdehyde via thiobarbituric acid which produces a pink-coloured product (125). However, it's use in a complex biological system is often criticised due to the non-specificity of thiobarbituric acid to react with other compounds such as simple and complex carbohydrates, protein oxidation products, and nucleic acid oxidation products (125). Due to the vast array of biomarkers and assays available, in addition to the limitations with indirect measurements of redox status which include non-specificity (125, 199, 276) and post-exercise time-course differences (280), it is recommended to measure multiple biomarkers at multiple time points and include measures of antioxidant activity when determining tissue redox homeostasis (172, 201).

2.1.3 Exercise-induced oxidative stress and metabolic health.

Previous studies have reported a mixture of increased and/or decreased biomarkers of oxidative stress and antioxidant activity after acute exercise (120). Equivocal findings likely result from variations in dietary intake, training status, exercise-intensity (44, 109, 118, 120), exercise-duration (42, 120, 201), exercise-mode (8, 42, 120), tissues sampled (42), sampling time points (42, 280), as well as the variety and volatility of the biochemical assays used (125). Nevertheless, the overarching consensus is that acute exercise elicits a transient increase in systemic and localized oxidative stress and antioxidant defence which, depending on the intensity and mode of exercise, can last for as long as 4 days after exercise (109, 120, 311).

Excessive ROS and/or oxidative stress induced through severe or extreme exercise regimes in humans can elicit cellular disturbances promoting muscular fatigue (342, 353), aberrant upregulation of endogenous antioxidant defences (101, 322), increased cardiovascular complications with ultra-endurance exercise (223), and impaired cognitive function (363) and exercise tolerance in murine animals (232). Aoi, et al. (14) reported that muscle damaging exercise in mice induced through downhill running increased skeletal muscle oxidative stress (TBARS), and resulted in 4-HNE mediated impairment of IRS-1 tyrosine phosphorylation, Akt phosphorylation and insulin stimulated glucose uptake 24 hours after-exercise. Thus, under certain conditions exercise-induced oxidative

stress has the potential to elicit a deleterious redox environment conducive to impaired exercise capacity and health (Figure 2.2).

The pathological effect of exercise-induced oxidative stress likely stems from secondary muscle damage leading to phagocytic infiltration into skeletal muscle (13) and subsequent generation of ROS (76, 301). In support, Nikolaidis, et al. (311) reported that muscle damaging exercise (75 lengthening knee flexions) significantly increased serum oxidative stress (TBARS, oxidized GSH and protein carbonyls) and serum antioxidant defence (catalase activity, uric acid, bilirubin and total antioxidant capacity) which lasted for up to 4 days after exercise. When a second identical bout of exercise was performed 3 weeks later, indices of muscle damage were lower which coincided with attenuation of the previously reported post-exercise redox status response (311). These studies provide evidence that muscle damaging exercise can elicit a transient state of elevated oxidative stress which in some cases may impair exercise performance, recovery, and metabolic health. However, this appears to occur independently from the transient and immediate increase in oxidative stress measured during and after exercise, and is likely attenuated with subsequent exercise-induced oxidative stress insults.

The majority of literature supports the idea that transient ROS production and/or oxidative stress elicited through regular exercise regimes (e.g. accustomed and/or non-extreme muscle damaging exercise) is beneficial and a necessary requirement for optimal physiological functioning and adaptation to physiological stress (346). Samjoo et al. (372) reported that 12 weeks of endurance training (2-3 session per week of 30-60 minutes cycling at 50% to 70% VO_{2peak}) in obese and sedentary men, decreased basal skeletal muscle and urinary markers of oxidative stress (4-HNE, protein carbonyls, and 8-isoprostane), increased basal skeletal muscle MnSOD protein abundance, and improved indices of glycaemic control. Thus, repetitive sessions of exercise-induced ROS (i.e. exercise training) can improve metabolic health through the upregulation of endogenous antioxidant defence and attenuation of basal chronic oxidative stress. Further support for the beneficial role of exercise-induced ROS can be found in human and animal studies that have reported antioxidant compounds to impair exercise mediated improvements in exercise capacity (156, 281), adaptive gene

expression and protein synthesis (156, 209, 327, 333, 411), upregulation of antioxidant defence (155, 156, 293, 314, 358, 411), improvements in cardiovascular health (150, 472), skeletal muscle inflammatory response and repair capabilities (281, 314), and insulin sensitivity (259, 358, 388, 429). Not all studies have reported the blunting of the aforementioned exercise-mediated adaptations (182, 236, 316, 478, 479), with some reports indicating enhanced exercise-induced adaptation with antioxidant supplementation (239, 305). Nevertheless, the general scientific consensus does not recommend the use of antioxidant supplementation for the improvement of exercise performance and skeletal muscle metabolic adaptation (158). It is now understood that exercise-induced ROS mediates cell proliferation, apoptosis, and differentiation, post-exercise inflammatory and immune responses. It also increases gene expression, transcription, and protein synthesis in important metabolic pathways that mediate mitochondrial biogenesis and endogenous antioxidant defence upregulation (106, 228, 346). An overview of the diverse role of oxidative stress in metabolic health is presented in Figure 2.2.

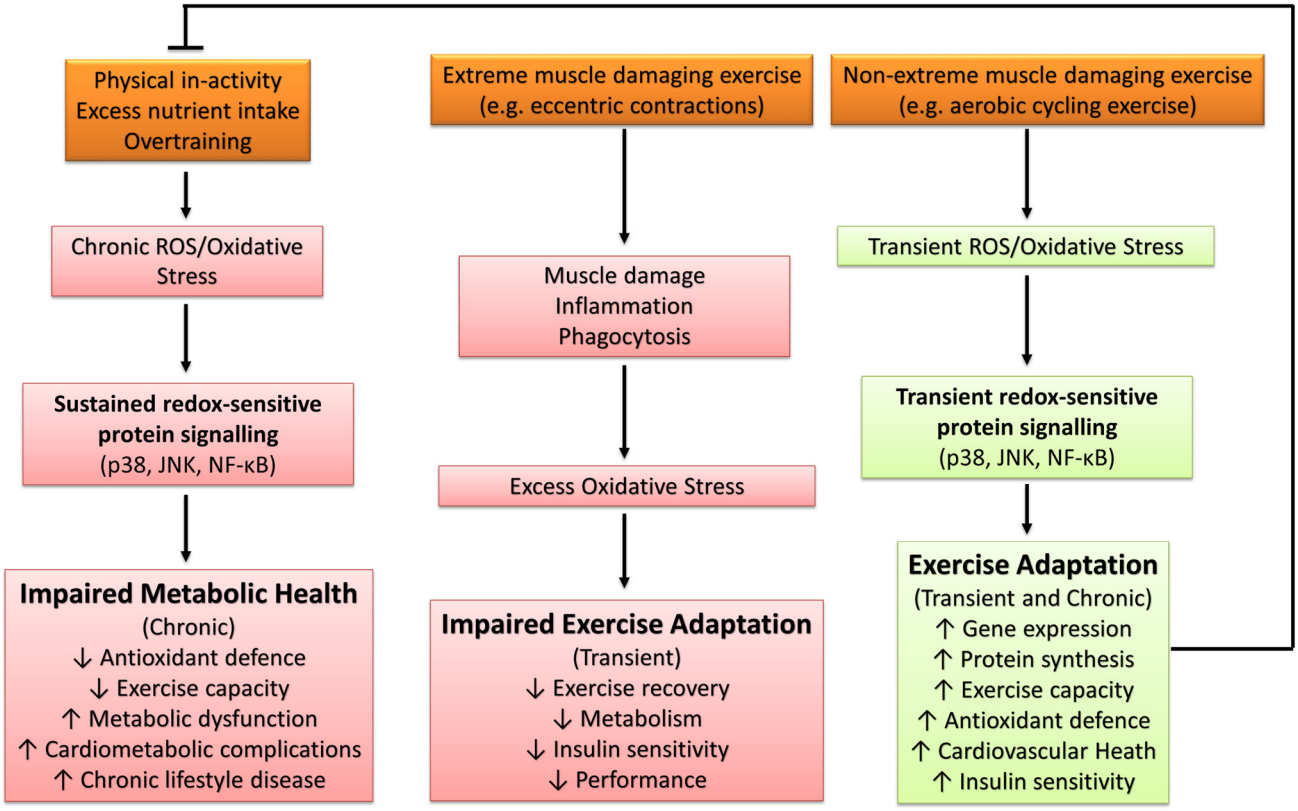

Figure 2.2: The influence of oxidative stress in health and disease.**P38:** p38 mitogen-activated protein kinases; **JNK:** c-Jun N-terminal kinases. **NF-κB:** Nuclear factor-κB.

2.1.4 Exercise-intensity and redox status.

Although few studies have directly compared the effects of different exercise-intensities, previous findings indicate that higher-intensity exercise (>75% maximal aerobic capacity, VO_{2max}) elicit greater systemic oxidative stress during and after exercise in humans compared to low-moderate intensity exercise (25% - 70% VO_{2max}) (162, 237, 254). Furthermore, higher-intensity exercise also elicits greater antioxidant defence (93, 323, 380, 435), however only a few studies exist and a limited number of exercise intensities have been investigated. Exercise-induced upregulation of endogenous antioxidant enzymes plays an important role in the protection against oxidative stress associated disease (52, 119, 122, 157, 346, 358, 436). As such, we recently investigated the effects of increasing exercise intensity, interspersed with short rest periods, on post-exercise changes in plasma redox status (323). In support of previous research, we confirmed that higher-intensity exercise significantly increases plasma biological antioxidant potential (BAP). We extended previous findings by reporting that in physically inactive but otherwise healthy males, short 5-minute cycling bouts at 40% and 55% of VO_{2max} had minimal impact on plasma BAP. In contrast, 5 minutes of cycling at 70%, 85% and 100% VO_{2max} significantly increased BAP, highlighting a potential exercise-intensity threshold for the transient induction of plasma antioxidant activity (Figure 2.3). Interestingly, plasma oxidative stress did not change with exercise. The acute short bouts of exercise may not have elicited sufficient ROS to overwhelm antioxidant defences in healthy males, a conclusion reported by others (380). Our findings, in addition to previous reports, indicate that exercise-intensity is an important modulator of exercise-induced ROS and/or antioxidant activity.

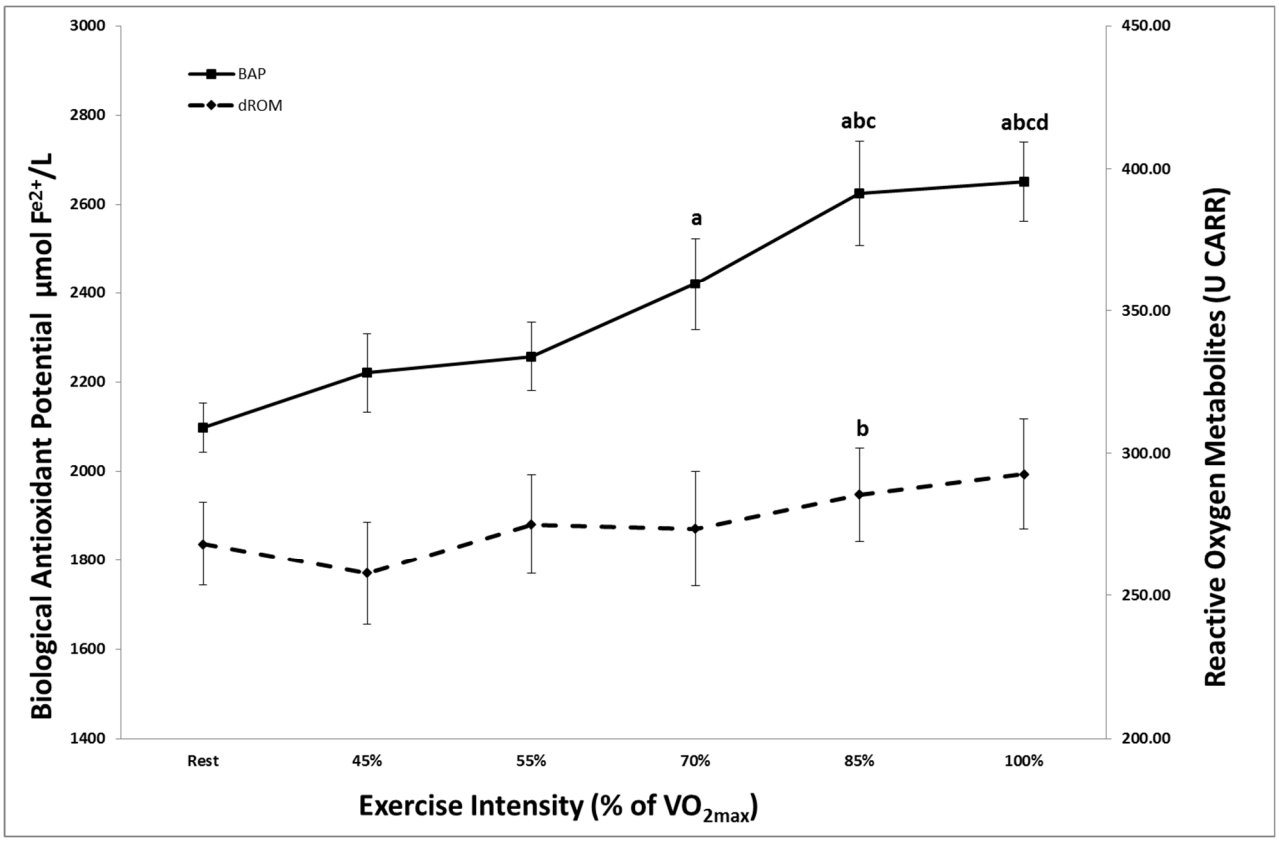

Figure 2.3: Exercise intensity and plasma reactive oxygen metabolites and biological antioxidant potential. Reactive oxygen metabolite (dROM) and biological antioxidant potential (BAP) responses at the completion of each exercise intensity bout. Values are mean ± SEM. [a]p<0.05 vs. rest, [b]p<0.05 vs. 40%, [c]p<0.05 vs. 55%, [d]p<0.05 vs. 70% of $VO2_{max}$. Replicated from Parker, et al. (323).

2.1.5 High-intensity interval-exercise and redox status.

Interest in HIIE has grown over the past decade (418). HIIE consists of repeated short intervals of high-intensity exercise interspersed with short passive or active recovery periods. Bogdanis, et al. (52) reported that a single session of HIIE consisting of 4 x 30 second cycling sprints, interspersed with 4-minute recovery periods, significantly increased blood protein carbonyls and TBARS at 30 minutes, 24 hours, and 48 hours after exercise. Likewise, antioxidant defence was also elevated with blood GPx increasing 24 and 48 hours after exercise; catalase increasing 30 minutes and 24 hours post-exercise; and total antioxidant capacity increasing 30 minutes, 24 and 48 hours after exercise. Fisher et al. (123) reported that acute HIIE consisting of 4 x 30 second cycling bouts at 90% W_{max}, interspersed with 4 minutes recovery, significantly increased plasma TBARS immediately after and 3 hours after exercise. Furthermore, lymphocyte enzymatic antioxidant activity of catalase, SOD and GPx activity increased immediately after

exercise, and SOD activity 3 hours after exercise. HIIE appears to be a potent stimulus for the transient induction of systemic oxidative stress and concomitant antioxidant defence. However, to the candidate's knowledge only one study has compared the effects of HIIE and continuous moderate-intensity exercise (CMIE) on post-exercise redox status (450).

Recently, Wadley, et al. (450) measured in untrained males the systemic redox status response to low-volume HIIE (LV-HIIE: 10 x 1 minutes at 90% VO_{2max}; 1 minute recovery intervals at 40% VO_{2max}; total time 19 minutes), and two work-matched steady state cycling protocols of high-intensity (80% VO_{2max}; 20 minutes) and moderate-intensity (60% VO_{2max}; 27 minutes). Findings indicated a similar increase in plasma lipid peroxidation and decrease in plasma protein carbonyls immediately after exercise, and no change in total antioxidant capacity, irrespective of the exercise protocol (450). These findings suggest a similar post-exercise redox status response between HIIE and CMIE, however blood biomarkers were not measured beyond 30 minutes post-exercise. Many oxidative stress and antioxidant biomarkers, including total antioxidant capacity, protein carbonyls, and the lipid peroxidation marker of TBARS, are reported to peak between 1 and 4 hours post-exercise (280). As such, further research comparing the effects of HIIE and CMIE on post-exercise redox status is warranted. A summary of research investigating the effect of a single session of HIIE on redox status is presented in Table 2.1.

Table 2.1: Summary of research investigating the effect of acute high-intensity and sprint interval-exercise on redox status in humans.

Reference	Participants	Exercise protocol	Time point	Oxidative Stress
Bogdanis, et al. (52)	8 Young Males Active Healthy	**SIE:** single session including 4 x 30s (4 min recovery periods)		**Blood/Plasma/Serum**
			Post-ex:	↑CAT, ↑TAC, ↑TBARS, ↑PC, **NC** GPX
			24h post-ex:	↑GPX, ↑CAT, ↑TAC, ↑TBARS, ↑PC
			48h post-ex:	↑GPX, ↑TAC, ↑TBARS, ↑PC, **NC** CAT
	After 3 weeks of high-intensity interval training			**Blood/Plasma/Serum**
			Post-ex:	**NC** GPX, ↑CAT, ↑TBARS, ↑PC, **NC** TAC
			24h post-ex:	↑GPX, ↑CAT, ↑TBARS, ↑PC, **NC** TAC
			48h post-ex:	↑GPX, ↑TBARS, **NC** PC, CAT and TAC
Canale et al. (59)	12 Young Males Trained Healthy	**No-exercise:** Rest **CMIE:** 60 min @ 70% HRR **HIIE:** 5 x 60s @ 100% W_{max} (225s recovery periods) **SIE:** 10 x 15s @ 200% W_{max} (116s recovery periods)		**Plasma/Serum**
			2h postprandial	All Ex: ↑H_2O_2, ↑MDA, ↑AOPP, ↓SOD, ↓CAT, **NC** GPX and Trolox
			4h postprandial	All Ex: ↑H_2O_2, ↑MDA, ↑AOPP, ↓SOD, ↓CAT, **NC** GPX and Trolox

		NOTE: Meal ingestion 1h after exercise		SIE: Main group effect ↑ Trolox
Fisher, et al. (123)	8 Young Males Active Healthy	**3 sessions of HIIE, 48 hours apart** **HIIE:** 4 x 30s @ 90% max anaerobic power (4 min recovery periods) Finished with 2min @ 15% max anaerobic power	Session 1. Post-ex: 3h post-ex: 24h post-ex: Session 2. Post-ex: 3h post-ex: 24h post-ex: Session 3. Post-ex: 3h post-ex: 24h post-ex: All time-points:	**Plasma (TBARS), Lymphocyte (antioxidant activity).** ↑TBARS, ↑CAT, ↑SOD, ↑GPX ↑SOD, **NC** TBARS, CAT and GPX **NC** TBARS, SOD, CAT, GPX ↑TBARS, ↑CAT, ↑SOD, **NC** GPX ↑TBARS, ↑SOD, **NC** CAT and GPX **NC** TBARS, SOD, CAT, GPX ↑SOD, ↑GPX, **NC** TBARS and CAT ↑SOD, **NC** TBARS, CAT, GPX **NC** TBARS, SOD, CAT, GPX Lymphocyte gene expression: **NC** CAT, CuZn-SOD, Mn-SOD, and GPX.
Tossige-Gomes et al. (423)	10 Young Males Active Healthy	**HIIE:** 8 x 1 min @ ~90-100% W_{max} (75s recovery periods)	Post-ex: 30min post-ex:	**Lymphocyte** **NC** TBARS, GSH, CAT and SOD ↑TBARS, ↓CAT, **NC** GSH and SOD
Wadley, et al. (450)	10 Young Males Active Healthy	**HIIE:** 10 x 1 min @ 90% VO_{2max} (1 min recovery periods) **High-intensity continuous:** 20 min @ 80% VO_{2max} **CE:** 27 min @ 60% VO_{2max}	Post-ex: 30min post-ex:	**Plasma** All ex: ↑LOOH, ↓PC, **NC**: TAC Al ex: **NC** LOOH, PC and TAC.
Morrison, et al. (293)	11 Young Males Healthy	**HIIE:** 10 x 4 min @ 90% VO_{2peak} (2 min recovery periods)	Post-ex:	**Skeletal muscle** ↑F 2 -isoprostanes (p=0.056) ↑Oxidised glutathione (p=0.058)

Abbreviations: **HIIE:** high-intensity interval exercise. **SIE:** sprint interval exercise. **WM:** work-matched. **CE:** continuous exercise. **CMIE:** continuous moderate-intensity exercise. **NC:** no change. ↑ significant increase compared to baseline or control. ↓ significant decrease compared to baseline or control. **Young:** participants 18-40 years old. **Middle-aged:** 40-65 years old. **Older**: >65 years old. **GPX:** glutathione peroxidase. **CAT:** catalase. **SOD:** superoxide dismutase. **TBARS:** Thiobarbituric acid reactive substances. **PC:** protein carbonyls. **MDA:** malondialdehyde. **AOPP:** advanced oxidation protein products. **GSH:** reduced glutathione. **MnSOD:** manganese superoxide dismutase. **CuZu-SOD:** copper/zinc-superoxide dismutase. **H_2O_2:** hydrogen peroxide. **LOOH:** lipid hydroperoxide. **HRR:** heart rate reserve.

Evidence provided indicates that acute exercise elicits a transient state of elevated ROS, oxidative stress and/or antioxidant defence, in an exercise-intensity dependant manner. This transient state of elevated oxidative stress in the hours following exercise may play an important role in exercise-mediated adaptations within skeletal muscle and the related benefits to metabolic health. However, the mechanisms responsible for ROS induced adaptation in skeletal muscle following exercise are unclear. It is proposed that the activation of redox-sensitive stress and mitogen activated protein kinase signalling pathways may play such a role (228).

2.2 Stress and mitogen activated protein kinase signalling.

Stress activated protein kinase (SAPK) and mitogen activated protein kinase (MAPK) signalling pathways include, but are not limited to, p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinases (JNK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and protein kinase C (PKC) (233, 320). For the purpose of this thesis, both MAPK and SAPK are collectively referred to as SAPK.

SAPK signalling pathways play an important signalling role in cellular proliferation, differentiation, survival, and cell death. Uncontrolled or sustained activation of SAPK signalling pathways are associated with the development and progression of cancer, neurodegenerative, and cardio-metabolic disease (217, 299, 421, 436). In contrast, controlled and/or transient SAPK activation is required for normal physiological functioning and reported to mediate many of the adaptations and health benefits received from regular exercise (228, 320).

SAPK pathways are activated through numerous stimuli involving hormones, growth factors, cytokines, agents acting through G protein-coupled receptors, transforming growth factors, pathogens and danger-associated molecular patterns, and physical and chemical stresses (210, 214, 233). Relevant to the current thesis however, is the inherent capacity of ROS to both directly and indirectly activate SAPK signalling pathways (34, 69, 87, 209, 275, 378).

2.2.1 ROS-induced SAPK signalling.

The direct oxidation of proteins on cysteine residues by ROS act as biological “switches” turning on the catalytic properties of numerous proteins and enzymes (283). Cysteine thiol oxidation produces sulfenic acids which form irreversible oxidation products or, in many cases, reacts to form reversible disulphide and sulfenamides bonds. These bonds can later be reduced via enzymes or compounds such as thioredoxin and glutathione, acting as an “off switch” and inhibiting protein function and enzymatic activity (222, 383). ROS induced SAPK signalling can occur through reversible oxidative modification processes that involve MAPK kinase kinases (MAP3K/MAP2Ks) (399). For example, apoptosis signal-regulated kinase 1 is activated via oxidative inactivation of thioredoxin, which leads to activation of the p38 MAPK and JNK signalling pathways (192,

368). MAPK phosphatases (MKPs), which maintain SAPK signalling pathways in an inactive state, are also susceptible to ROS induced oxidative modification on cysteine residues leading to phosphatase inactivation, degradation, and subsequent activation of SAPK signalling (114, 207, 303, 488). Additionally, SAPK activation can occur through ROS induced inactivation of glutathione S-transferases (1), tyrosine phosphorylation of protein kinase D (409), tyrosine and serine phosphorylation of upstream targets such as IκBα (231), and the interaction with growth factor and cytokine receptors (168, 222). Cross-talk also exists between SAPK signalling pathways, with activation of one pathway (e.g. JNK and p38 MAPK) often interacting with and activating other pathways (e.g. NF-κB) (205). Irrespective of the mechanisms, considerable research has reported increased SAPK signalling under conditions of elevated ROS production (34, 69, 87, 209, 275, 378).

2.2.2 Exercise-induced SAPK signalling.

The mechanical and physiological stress elicited by acute exercise provides a potent stimulus for the transient activation of SAPK signalling in human skeletal muscle in-part through increased ROS production (228). Exercise-induced SAPK signalling activate important skeletal muscle transcription factors and coactivators which include proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), activating transcription factor 2, myocyte-enhancing factor 2, c-jun, c-fos, p53, and Elk-1 (16, 65, 106, 130, 167, 209, 216, 228, 252, 398, 420). Exercise-induced SAPK signalling also promotes the upregulation of antioxidant defences such as SOD, inducible nitric oxide synthase, gamma-glutamylcysteine synthetase, GPx, catalase, redox effector factor-1, nuclear factor erythroid 2-related factor 2, antioxidant response element, and Kelch-like ECH-associated protein 1 (29, 106, 209, 228, 243, 333, 385, 442, 454, 484).

Evidence supporting a role for exercise-induced ROS and SAPK signalling in exercise adaptation is primarily derived from research manipulating the redox environment to attenuate or enhance the exercise-induced ROS and protein signalling response. Henriquez-Olguin et al. (181) recently reported that inhibition of the ROS producing enzyme complex NADPH oxidase 2 in rats, attenuates the exercise-induced skeletal muscle phosphorylation of p38 MAPK and NF-κB p65, and gene expression of MnSOD, GPx, citrate synthase and mitochondrial

transcription factor A (mtTFA). Similar findings have also been published using ROS inhibitors (e.g. antioxidant supplementation) in animals (154, 155, 209). Using a novel approach, Strobel et al. (410) reported that increased exercise-induced oxidative stress via skeletal muscle glutathione depletion in rats resulted in greater PGC-1α gene expression. In humans, antioxidant supplementation attenuates exercise-induced activation of p38 MAPK, NF-κB p65 and JNK protein signalling, and gene expression of SOD isoforms in skeletal muscle (155, 281, 333). Chronic inhibition of exercise-induced oxidative stress also impairs the training induced upregulation of PGC-1α, nuclear respiratory factor (NRF)-1, and mtTFA in rats (209).

Not all studies report a clear role for redox-sensitive protein kinase signalling in exercise adaptation. Wadley et al. (451) reported similar PGC-1α, NRF-2 and SOD gene expression after exercise with allopurinol treatment in rats despite decreased p38 MAPK phosphorylation and mtTFA gene expression. In addition, chronic allopurinol treatment was reported to have no effect on the training induced upregulation of PGC-1α, mtTFA, cytochrome c, citrate synthase, and β-hydroxyacyl-CoA dehydrogenase (451). In humans, Morrison, et al. (293) reported vitamin C and E supplementation to have little effect on exercise-induced gene expression of PGC-1α, mtTFA, and PGC related coactivator, or training induced improvements in VO_{2peak}, citrate synthase activity, and expression of cytochrome oxidase subunit 4. However, SOD activity and protein abundance of SOD and mtTFA were attenuated by vitamin C and E supplementation (293). A summary of key findings from research investigating redox manipulation, exercise and SAPK signalling are reported in Table 2.2.

It is possible that the large discrepancy in findings are due to the large variations in the methods and/or compounds used to modulate exercise-induced ROS, with many effective animal methods having little to no effect in humans (158, 170, 270, 271, 302, 395). Nevertheless, evidence provided so far indicates a role for redox-sensitive SAPK signalling in skeletal muscle adaptation. However, the specific roles and to what degree SAPK signalling plays in exercise adaptation requires further investigation.

2.2.3 Exercise-intensity and SAPK signalling.

The p38 MAPK signalling pathway is redox-sensitive (209), upregulates endogenous antioxidant defence (385), and plays a critical role in mitochondrial biogenesis and angiogenesis (4, 117, 344). As such, the effect of acute exercise on p38 MAPK signalling has received the greatest attention out of the SAPK family. Previous research indicates that both CMIE (249) and HIIE are capable of eliciting p38 MAPK phosphorylation in the acute post-exercise recovery period (142, 248, 482). Likewise, sprint interval exercise (SIE; 4 x 30 second cycling sprints, interspersed with 4 minutes recovery) increases p38 MAPK phosphorylation immediately after exercise, PGC1-α gene expression 3 hours after exercise (142, 248), and increases multiple measures of mitochondrial capacity 24 hours after exercise (248). These studies provide evidence that even a single bout of HIIE/SIE can transiently increase p38 MAPK signalling and elicit mitochondrial biogenesis 24 hours post-exercise. However, whether higher-intensity exercise elicits greater post-exercise p38 MAPK signalling when compared to CMIE is less clear (29, 79, 105).

Table 2.2: Summary of key findings from research investigating the effect of redox status manipulation on acute exercise-induced protein signalling and molecular markers of skeletal muscle adaptation.

Reference	Participants	Exercise	Redox manipulation	Time point	SAPK signalling	Markers of skeletal muscle adaptation
Gomez-Cabrera, et al. (155)	25 Adults Trained Healthy	Marathon	Allopurinol (n=14) Placebo (n=11)	Post-ex.	Placebo only: ↑ lymphocyte NF-κB p50 activity.	
Gomez-Cabrera, et al. (154)	15 male Wistar rats	Exhaustive treadmill exercise	Rest (n=5) Exercise (n=5) Exercise + Allopurinol (n=5)	Post-ex.	Placebo only: ↑ p-p38 MAPK, NF-κB activity.	Placebo only: ↑ Mn-SOD, iNOS and eNOS mRNA.
Henriquez-Olguin, et al. (181)	20 male BalbC mice	Swimming exercise	Apocynin (n=10) Vehicle (n=10)	Post-ex.	Apocynin: attenuated p-p38 MAPK and p-NF-κB p65	Apocynin: **Attenuated** Mn-SOD, GPX, CS and mtTFA mRNA.
Kang, et al. (209)	18 Female Sprague–Dawley rats	Exhaustive treadmill exercise	Allopurinol (n=9) Vehicle (n=9)	Post-ex.	Allopurinol: attenuated p-p38 MAPK, p-IκBα, NF-κB DNA binding,	Allopurinol: attenuated PGC-1α, p-CREB, NRF-1, mtTFA content.
Michailidis, et al. (281)	10 Young Males Active Healthy	300 unilateral eccentric leg repetitions	Cross-over: N-acetylcysteine and placebo	2h post-ex. 2d post-ex. 8d post-ex.	Both: ↑ p-p38 MAPK **NC** p-NF-κB p65. NAC: **greater** p-p38 MAPK. **Attenuated** p-NF-κB p65. Both: **NC** p-NF-κB p65. NAC: **Attenuated** p-p38 MAPK.	Both: ↑ p-Akt^{Ser473}, p-p70S6K^{Thr389} and p-rpS6. **NC** MyoD. Muscle function impaired (mean torque) Both: ↑ p-Akt^{Ser473}. **NC** MyoD. NAC: **Attenuated** mTOR^{Ser2448}, p-p70S6K^{Thr389} and p-rpS6. Muscle function impaired. NAC: **Attenuated** p-Akt^{Ser473}, mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-rpS6 and MyoD. Placebo only: Muscle function completely recovered.
Petersen, et al. (333)	8 Young Males Trained Healthy	45 mins @ 71% VO_{2peak} followed by 92% VO_{2peak} to fatigue	Crossover: N-acetylcysteine and Saline infusion	Post-ex. (45 mins @ 71% VO_{2peak}) Post-ex. (fatigue)	Both: ↑ p-p38 MAPK, ↓ IκBα**. NC** p-NF-κB p65. NAC: **Attenuated** p-JNK NAC: **Attenuated** p-JNK**,** ↓ p-NF-κB p65.	Both: ↑ PGC-1 α mRNA NAC: **Attenuated** Mn-SOD mRNA Both: **NC** PGC-1 α mRNA and Mn-SOD mRNA

Strobel, et al. (410)	Male Wistar rats	Exhaustive treadmill exercise	Diethyl maleate (DEM) and controls	Post-ex. 4h post-ex.	Both ↑ p-p38 MAPK. Not measured	Not measured Both: **NC** NRF-2 DEM: greater ↑ PGC-1α mRNA. **Attenuated** GPx mRNA
Trewin, et al. (429)	7 Young Adults Active Healthy	55 mins at 65% VO_{2peak} followed by 5 mins @ 85% VO_{2peak}	Crossover: N-acetylcysteine and Saline infusion	Post-ex.	NAC: ↓ p-p38 MAPK	Both: ↑ p-p70S6K^{Thr389} and p-rpS6
Wadley, et al. (451)	Male Sprague-Dawley rats	Treadmill exercise	Allopurinol or placebo	Post-ex. 4h post-ex.	Allopurinol: **Attenuated** p-p38 MAPK Not measured	Not measured Both: ↑ mtTFA, NRF-2, PGC-1α, GLUT-4, MnSOD and ecSOD mRNA Allopurinol: **Attenuated** mtTFA mRNA

*****Molecular response in skeletal muscle unless otherwise noted. **NC:** no change compared to baseline or control. ↑ significant increase compared to baseline or control. ↓ significant decrease compared to baseline or control. **iNOS:** inducible nitric oxide synthase. **eNOS:** endothelial nitric oxide synthase. **CS:** Citrate synthase. **mtTFA:** mitochondrial transcription factor A. **NRF-1/2**: nuclear respiratory factor-1 and 2. **MyoD:** Myogenic determination factor. **PGC-1 α:** peroxisome proliferator-activated receptor gamma coactivator 1 α. **PRC:** PGC related coactivator. **Young:** participants 18-40 years old. **Middle-aged:** 40-65 years old. **Older**: >65 years old. **Active:** recreationally active. **T:** trained. **GPx:** glutathione peroxidase. **MnSOD:** manganese superoxide dismutase. **EcSOD:** extracellular superoxide dismutase. **CuZu-SOD:** copper/zinc-superoxide dismutase. **H_2O_2:** hydrogen peroxide. **LOOH:** lipid hydroperoxide. **HRR:** heart rate reserve.

Egan, et al. (105) reported similar p38 MAPK phosphorylation in healthy humans immediately after CMIE (approximately 36 minutes of cycling at 60% $VO2_{peak}$) and work-matched high-intensity continuous cycling (approximately 70 minutes at 80% $VO2_{peak}$), suggesting little involvement of exercise-intensity on p38 MAPK signal transduction. Interestingly, Combes et al. (81) recently reported greater phosphorylation of p38 MAPK, and upstream PGC-1α regulatory pathways AMPK and CaMKII, with intermittent cycling exercise (30 x 1-min intervals at 70% $VO2_{peak}$; 1 minute recovery periods) compared to work and intensity matched continuous exercise (30 minutes at 70% $VO2_{peak}$). It was proposed that increased cellular metabolic disturbances (i.e. oscillations of the cytosolic NADH/NAD+ redox state (245)) elicited through intermittent exercise played a larger role in p38 MAPK signalling compared to exercise volume or intensity. In contrast however, both HIIE (6 x 3-min at 90% VO_{2max}; 3-minute recovery periods at 50% VO_{2max} (29)), and SIE (4 x 30 second Wingate tests; 4 min passive recovery periods (79)) were reported to elicit similar post-exercise p38 MAPK phosphorylation compared to work-matched continuous exercise. It is possible that the metabolic disturbances elicited by HIIE were too few (81), and/or the intensity of the continuous exercise protocols of 50 minutes running at 70% VO_{2max} (29), and 4 minutes of intense cycling (79), were too high to elicit significantly different skeletal muscle p38 MAPK phosphorylation patterns. Nevertheless, evidence provided here supports HIIE as a potent stimulus for eliciting post-exercise p38 MAPK signalling, however research comparing HIIE and CMIE are contradictory and thus further research is warranted (Table 2.3).

Table 2.3: Summary of research investigating the effect of acute high-intensity interval exercise and sprint interval exercise on stress and mitogen activate protein kinase signalling in humans.

Reference	Participants	Exercise protocol	Time point	SAPK
Bartlett, et al. (29)	10 Young Males Active Healthy	**HIIE:** 6 x 3 min @ 90% VO_{2max} (3 min recovery periods) **WM-CE:** 50 min @ 70% VO_{2max}	Post-ex: 3h post-ex:	All Ex: ↑ p-p38 MAPK All Ex: **NC p**-p38 MAPK
Cochran, et al. (79)	8 Young Males Active Healthy	**SIE:** 4 x 30s all-out sprints (4 min recovery periods) **WM-CE:** total-work matched to SIE as single all-out effort.	Post-ex:	All Ex: ↑ **p**-p38 MAPK
Combes, et al. (81)	9 Young Males Active Healthy	**HIIE:** 30 x 1 min @ 70% W_{max} (1 min recovery periods) **WM-CE:** 30 mins @ 70% W_{max}	Post-ex: 3h post-ex:	HIIE: ↑ **p**-p38 MAPK All Ex: **NC p**-p38 MAPK
Gibala, et al. (142)	6 Young Males Active Healthy	**SIE:** 4 x 30s all out (4 min recovery periods)	Post-1st sprint: Post-ex: 3h post-ex:	**NC p**-p38 MAPK ↑ **p**-p38 MAPK **NC p**-p38 MAPK
Little, et al. (248)	8 Young Adults Active Healthy	**SIE:** 4 x 30s all out (4 min recovery periods)	Post-ex: 3h post-ex: 24h post-ex:	↑ **p**-p38 MAPK **NC p**-p38 MAPK ↑ **p**-p38 MAPK
Yu, et al. (482)	6 Young Adults Trained 4 Young Adults Inactive	**Trained HIIE protocol:** 8 x 5 min **@** ~85% VO_{2max} (1 min recovery periods) **Untrained HIIE protocol:** 4 x 5 min **@** ~85% VO_{2max} (1 min recovery periods)	Post-ex:	All ex: ↑ **p**-p38 MAPK Greater ↑ **p**-p38 MAPK in untrained.

HIIE: high-intensity interval exercise. **SIE:** Sprint interval exercise. **WM:** work-matched. **CE:** Continuous exercise. **CMIE:** continuous moderate-intensity exercise. **NC:** no change compared to baseline or control. ↑ significant increase compared to baseline or control. ↓ significant decrease compared to baseline or control. **Young:** participants 18-40 years old. **Middle-aged:** 40-65 years old. **Older**: >65 years old. **T2DM:** type 2 diabetes mellitus.

A single bout of treadmill running (25 m/min, 5% grade, for 1 h or until exhaustion) significantly elevates NF-kb activity in rat skeletal muscle immediately after exercise, and 1, 2, 4 and 24 hours after exercise (204). Others have also reported increased NF-kb activity in rodent skeletal muscle after acute exercise (183, 184, 209). On the other hand, evidence in human skeletal muscle is equivocal. Durham et al. (103) reported decreased NF-κB activity immediately after resistance exercise, returning to baseline 1-hour post-exercise. Likewise, NF-κB p65 phosphorylation is not significantly different immediately after continuous cycling (71% VO_{2peak} for 45 min) or cycling to fatigue (92% VO_{2peak}) (333). In contrast, NF-κB activity in human peripheral blood mononuclear cells (PBMCs) is increased after a single 30 second Wingate test, peaks approximately 60 minutes after exercise, and remains elevated for up to 120 minutes post-exercise (90). Likewise, high-intensity continuous cycling (60 minutes at 80% of VO_{2max}) significantly increased NF-κB activity in peripheral blood lymphocytes

immediately after exercise (444). It is possible that post-exercise skeletal muscle sampling time-points in human studies may not have captured the time-dependent increase in exercise-induced NF-κB phosphorylation and/or activity. In healthy men, increased activation of the NF-κB signalling pathway in PBMCs occurs in an exercise-intensity dependant manner after treadmill running at 40, 60, 80, and 100% of HR reserve, however the duration of the exercise bouts, potential rest periods, and whether the exercise bouts were performed on different days were not disclosed (220). To the author's knowledge research has yet to investigate the effect of a single session of HIIE on NF-κB signalling in human skeletal muscle.

Continuous cycling exercise in healthy humans for 60 minutes (70% VO_{2max}), and for 45 min (71% VO_{2peak}) followed by cycling until fatigue (92% VO_{2peak}), increases JNK activity and/or phosphorylation in skeletal muscle immediately post-exercise (16, 333). Although human research has yet to investigate exercise intensity and post-exercise JNK signalling, in situ contraction of rat skeletal muscle suggest that the magnitude of mechanical stress (peak muscle tension) applied to the muscle is a strong predictor of contraction induced JNK phosphorylation (268).

From the evidence presented, SAPK signalling is activated by a single session of exercise. However, the role of exercise-intensity on post-exercise SAPK signalling is contradictory with only few studies conducted in humans. Considering the important role of JNK, NF-κB, and p38 MAPK signalling in skeletal muscle contraction induced gene transcription and adaptation (228), further research investigating the effect of HIIE on post-exercise SAPK signalling is warranted.

2.3 Glycaemic control.

ROS are readily induced through skeletal muscle contraction and activate redox-sensitive signalling pathways implicated in glucose metabolism (25, 34, 308, 321, 397, 420). However, prior to discussion of the role of redox biology in glycaemic control, an overview of glycaemic control and the influence of exercise is warranted.

2.3.1 Glucose homeostasis.

Glucose provides one of the main sources of fuel for cellular metabolism. The long-term maintenance of a steady supply of glucose plays an important physiological role in the survival of many species. Sustained elevation of glucose (i.e. hyperglycaemia) or low glucose concentrations (i.e. hypoglycaemia) creates a potentially deleterious environment that can impair cellular function and survival (129). Complex organisms have evolved an intricate and dynamic regulatory system that maintains glucose homeostasis to ensure adequate supply to meet demand. This system includes the interaction between intestinal glucose absorption, gluconeogenesis, and glycogenolysis (412). Under fasted physiological conditions, blood glucose levels are primarily maintained through pancreatic α-cell secretion of glucagon and the inhibition of pancreatic β-cell insulin secretion (129). Glucagon promotes the conversion of glycogen into glucose, increasing hepatic glucose output and a subsequent elevation in blood glucose. On the other hand, insulin secretion lowers blood glucose primarily through binding of insulin to insulin receptors on target tissues (e.g. muscle, fat and the liver), activating the canonical insulin signalling cascade and stimulating glucose transport across the plasma membrane; a biological process commonly referred to as insulin stimulated glucose uptake (30, 127, 421). Insulin secretion can also lower blood glucose through the direct and indirect inhibition of hepatic glucose production (148).

2.3.2 Insulin stimulated glucose uptake.

During conditions of elevated substrate availability, for example a glucose load from a meal, elevated blood glucose and to a lesser extent lipid and amino acid concentrations, are sensed by β-cells resulting in the secretion of insulin to maintain glucose homeostasis (379, 412). Under normal physiological conditions, insulin binds to the extracellular α-subunit of the insulin receptor promoting auto-phosphorylation of the transmembrane β-subunit on tyrosine residues 1158, 1162, and 1163 (462). Scaffolding proteins including Shc adapter protein isoforms, signal-regulatory protein family members, Gab-1, Cbl, adapter protein with a PH and SH2 domain, and insulin receptor substrates (IRS) are bound and tyrosine residues phosphorylated to promote subsequent binding to phosphatidylinositol-3 kinase (PI3K) (331, 463). Activation of PI3K generates

phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) that docks to and subsequently induces membrane translocation of the serine/threonine kinase Akt. PIP_3 activation of phosphoinositide-dependent kinase-1 (PDK1) and the Rictor/mTOR complex 2 lead to dual phosphorylation of Akt on serine 473 and threonine residue 308 promoting subsequent activation of Akt kinase (6, 19, 127, 375). Increased Akt activity elicits phosphorylation of Akt substrate 160 (AS160; also known as TBC1D4), promoting GTP loading and activation of Rabs releasing GLUT4 vesicles from intracellular compartments and promoting GLUT4 plasma membrane docking to facilitate glucose uptake (190). The protein TBC1D1 also functions as a Rab-GAP protein and has recently been identified as a key mediator of exercise-induced (contraction) and insulin stimulated glucose uptake (9, 408). The homology of TBC1D1 and AS160 are similar and share an almost identical GAP domain. Although TBC1D1 and AS160 share an overlap in function (359), this thesis focuses on AS160.

Skeletal muscle and adipose tissue are the two major sites for insulin stimulated glucose uptake, as such the insulin-sensitive glucose transporter isoform, GLUT4, is largely expressed in these tissues (189, 198). Akt not only promotes GLUT4 translocation, but also facilitates glycogen synthesis via inhibitory phosphorylation of glycogen synthase kinase 3 (GSK-3) on Ser23 (GSK-3α) and Ser9 (GSK-3β) (464). PIP_3 and PDK-1 also activate atypical PKC isoforms ζ and λ which are reported to facilitate GLUT4 vesicle trafficking and glucose uptake (128, 304, 404). A summary of the canonical insulin signalling pathway is presented in Figure 2.4.

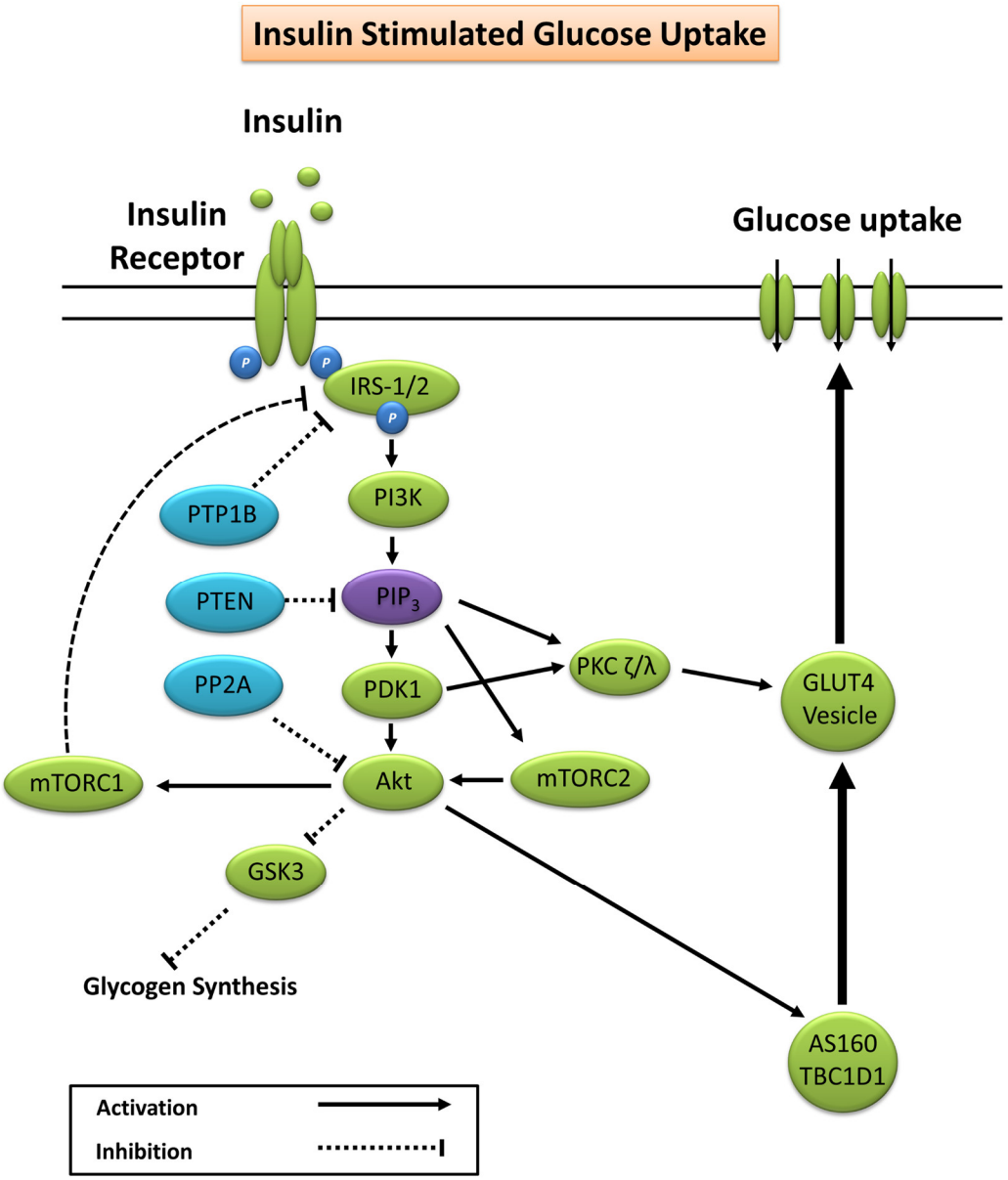

Figure 2.4: Primary signalling pathways involved in insulin stimulated glucose uptake. Abbreviations: **Akt**: protein kinase B; **AS160:** Akt substrate of 160 kDa; **GLUT4:** glucose transporter 4. **GSK3:** glycogen synthase kinase 3; **IRS-1/2:** insulin receptor substrate 1 and 2; **mTORC1/2:** mechanistic target of rapamycin complex 1/2; **PDK1:** phosphoinositide-dependent kinase-1; **PI3K:** phosphatidylinositol-3 kinase; **PIP3:** phosphatidylinositol (3,4,5)-trisphosphate; **PKC**: protein kinase C; **PP2A**: protein phosphatase 2; **PTEN:** phosphatase and tensin homolog; **PTP1B:** protein-tyrosine phosphatase 1B; **TBC1D1**: TBC1 domain family member 1.

2.4 The effect of exercise on glycaemic control.

2.4.1 Glucose uptake during exercise.

Glucose uptake during exercise occurs in an exercise-intensity and exercise-duration dependent manner, which depends largely on a combination of increased glucose delivery, glucose transport, and glucose metabolism (356, 452). Increased trafficking of GLUT4 to the plasma membrane during exercise occurs through cellular detection of changes in Ca^{2+} concentration (203, 469), changes in the energy charge of the cell (39, 55, 175, 406), remodelling of the actin cytoskeleton via GTPase Rac1 (413), and fibre type specific mediation of nitric oxide synthase (278). The primary protein signalling pathways include contraction-induced activation of calcium (Ca^{2+})/calmodulin-dependent kinase (CaMK), atypical PKC, CaMK phosphatase calcineurin, adenosine monophosphate-activated protein kinase (AMPK), Akt, and mitogen activated protein kinases (178, 228).

Although increased calcium release from contracting muscle stimulates CaMK and calcium/calmodulin-dependent protein kinase kinases (203, 469), the primary pathway suggested to mediate glucose uptake during exercise is through the energy sensing signalling pathway of AMPK (356). The binding of AMP and ADP, and decreased binding of ATP (174), to AMPK α and γ sub-units during exercise causes AMPK activation allosterically and by phosphorylation on threonine residue 172 (39, 55, 175, 406). Exercise induced AMPK, and to a lesser extent Ca^{2+} signalling pathways, elicits GLUT4 translocation and subsequent glucose uptake through phosphorylation and inactivation of the convergent glucose uptake signalling protein AS160 (229, 230). Animal studies have reported skeletal muscle knockout and inhibition of AMPK markedly reduces exercise-induced AMPK activity, glucose uptake and AS160 signalling (134). A summary of contraction induced glucose uptake during exercise is presented in Figure 2.5.

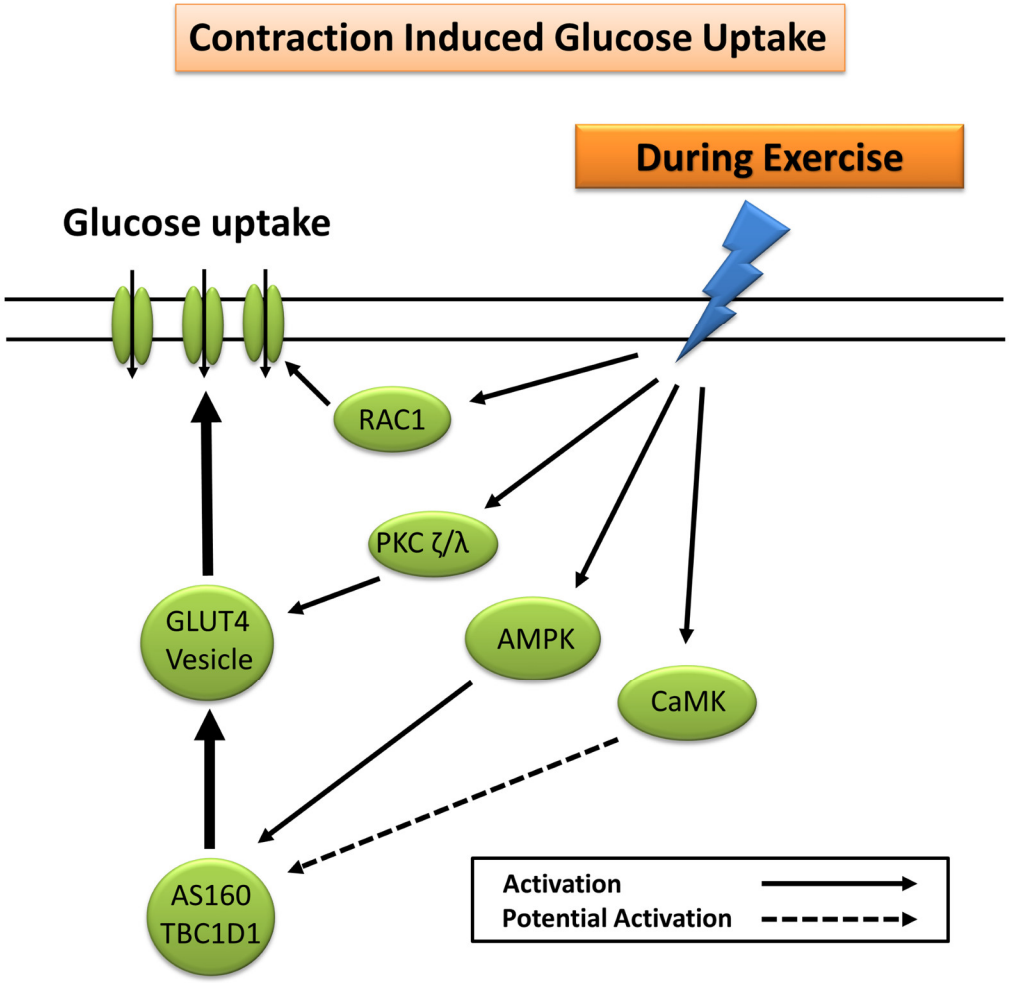

Figure 2.5: Primary signalling pathways involved in contraction induced glucose uptake. Abbreviations: **AMPK:** 5' adenosine monophosphate-activated protein kinase. **AS160:** Akt substrate of 160 kDa; **CaMK:** ca2+/calmodulin-dependent protein kinase; **GLUT4:** glucose transporter 4. **PKC:** protein kinase C; **RAC1**: ras-related C3 botulinum toxin substrate 1. **TBC1D1**: TBC1 domain family member 1.

2.4.2 Exercise-intensity and glycaemic control.

Glucose uptake during exercise is maintained in insulin resistant populations (221, 452). In contrast, basal and post-exercise insulin stimulated glucose uptake are impaired and contribute to the development of chronic disease (51, 282, 448). Regular exercise improves glycaemic control in healthy and clinical populations, however the effects of modifying exercise-intensity are equivocal (54, 82, 200, 251, 396). Reviews of the literature initially reported minimal relationship between exercise-intensity and improvements in glycaemic control (HbA1c, fasting glucose and insulin, postprandial glucose, and direct and indirect measures of

insulin sensitivity) in both healthy populations (82) and patients with T2DM (82, 396). More recently, meta-analysis' have reported the potential for high-intensity exercise to elicit greater improvements in some measures of glycaemic control in healthy and clinical populations (200, 251). Nevertheless, large variations in study design, population demographics, methods for measuring insulin sensitivity, different exercise intensities and durations, length of training, training compliance, and/or exercise modes complicate comparisons between studies. Certainly, research directly comparing the effects of high-intensity exercise with moderate-intensity exercise support the beneficial role of higher-intensity exercise (141, 200, 251).

2.4.3 High-intensity interval training and glycaemic control.

Karstoft et al. (211) reported that a 4-month training program of intermittent walking (5 sessions/week) improves insulin sensitivity in patients with type 2 diabetes to a greater extent than continuous walking exercise that was matched for energy expenditure and mean training intensity. Increased metabolic disturbances, elicited through intermittent exercise, were suggested to play a large role in the enhancement of insulin sensitivity. Certainly, accumulating evidence indicates that HIIE is a potent stimulus for improving exercise capacity and performance, and cardiometabolic health including glycaemic control (141, 251, 348, 460). These cardiometabolic health benefits are reported to be comparable, and in some cases greater than traditional CMIE (81, 141, 144, 246, 248, 250, 348, 460).

Mitranun et al. (285) recently reported that 12 weeks of HIIE treadmill running (3 sessions per week, 4-6 x 1 min bouts at 80%-85% VO_{2peak}) in patients with type 2 diabetes improved HbA1c levels, aerobic capacity, flow-mediated dilation, the glycoprotein Von Willebrand factor, decreased erythrocyte oxidative stress (TBARS), and increased erythrocyte antioxidant capacity (glutathione peroxidase), to a greater extent than energy-matched CMIE (continuous treadmill running at 60%-65% VO_{2peak}). HIIE and/or sprint interval exercise (SIE) that is lower in volume are also effective for improving glycaemic control (80, 141, 147, 391). For example, 12 weeks of low-volume SIE (3 x 20 second 'all-out' cycling sprints; 2 minute recovery periods) in overweight and inactive men improves aerobic capacity, maximum workload, insulin sensitivity, GLUT4 protein content,

and markers of mitochondrial biogenesis to a similar extent as CMIE (45 minutes of cycling at ~70% HR_{max}) (145). Even 2 weeks of LV-HIIE (10 x 1 min at ~60% of peak power, 1 min recovery periods; 3 sessions per week) is enough to increase peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), GLUT4 protein content, and insulin sensitivity (186). Importantly, an improvement in glycaemic control can be seen for up to 48 hours after a single bout of exercise (247, 330, 420, 429, 435, 437).

2.4.4 Acute high-intensity interval exercise and glycaemic control.

Previous studies exploring acute SIE (4 x 30 second all-out sprints, 4 - 4.5 minute recovery periods) reported no effect on insulin sensitivity, as measured by oral glucose tolerance test and the hyperinsulinaemic-euglycaemic clamp, 24 hours and 72 hours after exercise (354, 466). Surprisingly, an extended sprint work-matched to SIE improved insulin sensitivity 24 hours after exercise (466). However, questions have been raised about the practical implications, and limitations, of measuring insulin sensitivity through laboratory based methods which may not represent functional improvements in glycaemic control under free-living conditions (284). The direct measurement of insulin sensitivity via the hyperinsulinaemic-euglycaemic clamp is valid, reliable, and considered the gold standard method (96). However, the infusion of supraphysiological levels of insulin during the clamp do not accurately reflect insulin action and glucose dynamics under physiological conditions (295). Dynamic tests such as the oral glucose and intravenous glucose tolerance tests, and simple surrogate indexes such as the homeostasis model assessment of insulin resistance (HOMA-IR), may not always reflect functional improvements in glycaemic control (284).

A study by Mikus, et al. (284) highlighted the importance of using continuous glucose monitoring (CGM) under-free living conditions to reflect functional changes in glycaemic control. The author reported no change in glycaemic control in patients with T2DM as measured by both oral glucose tolerance test and fasting blood glucose concentrations after 7 days of CMIE (60 minutes at 60-75% HRR). However, 24-hour CGM measurements revealed significant improvements in the postprandial glucose response to standard meals, and improvements in the frequency, magnitude and the duration of daily glycaemic

excursions (284). As such, CGM may be a clinically important instrument to assess glucose control under free living conditions.

Impaired glycaemic variability is reported to contribute to the development of diabetic complications and is associated with increased mortality (67, 110, 473). Manders et al. (260) reported that a single session of CMIE (60 min at 35% W_{max}) in participants with T2DM significantly decreased 24-hour average glucose levels and the prevalence of hyperglycaemia. Surprisingly, work-matched high-intensity continuous exercise (30 min at 70% W_{max}) failed to elicit improvements in glycaemic control (260). This is however in contrast with previous reports that acute HIIE (10 x 60 second cycling bouts at ~90% HR_{max}; 1 min recovery periods) in patients with type 2 diabetes improves 3-hour postprandial glycaemia and 24-hour average glucose levels (144). Furthermore, recent research in overweight and obese adults reported greater improvements in 24-hour glycaemic control and the postprandial glucose response to breakfast the day after HIIE (10 x 1-min at 90% W_{peak}; 1 min recovery periods at 15% W_{peak}) compared to work matched CMIE (30 min at 35% W_{peak}) (247).

Current evidence indicates that acute HIIE is an effective exercise stimulus for enhancing 24-hour post-exercise glycaemic control and are summarised in Table 2.4. However, previous studies have only compared the effects of HIIE and SIE to work-matched continuous exercise protocols (247, 466), not included a continuous exercise protocol comparison group (144, 354), or have not implemented functional measures of 24-hour post-exercise glycaemic control (354, 466). Consequently, whether acute lower-volume HIIE provides similar benefits in 24-hour post-exercise glycaemic control to that of CMIE is unknown.

Table 2.4: Summary of research investigating the effect of acute high-intensity interval exercise and sprint interval exercise on glycaemic control in humans.

Reference	Participants	Exercise protocol	Time point	Glycaemic Control
Gillen, et al. (144)	7 Middle-aged Adults Inactive T2DM	**HIIE:** 10 x 60s @ ~90% W_{max} (60s recovery periods)	Up to 24h post-ex.	**CGM measures** ↓3h postprandial AUC ↓postprandial avg glucose ↓postprandial peak ↓hyperglycaemia
Little, et al. (247)	10 Young Adults Inactive Overweight/obese	**HIIE:** 10 x 60s @ ~90% W_{max} (60s recovery periods) **WM-CMIE:** 30 min @ 35% W_{max}	Up to 24h post-ex.	**CGM measures** All ex: **NC** 24h avg glucose, MAGE, SD, postprandial lunch. All ex: ↓ post-prandial dinner and breakfast (greater ↓ with **HIIE**)
Richards, et al. (354)	9 Young Adults Active and Inactive Healthy	**SIE:** 4 x 30 seconds all-out efforts cycling (4 min recovery periods)	At 72h post-ex.	**Insulin clamp** **NC** insulin sensitivity
Whyte, et al. (466)	10 Young males Inactive Overweight/obese	**SIE:** 4 x 30 seconds all-out cycling efforts (4.5 min recovery periods) **ES:** single bout work-matched to SIE.	At 24h post-ex.	**OGTT** **SIE: NC** insulin sensitivity **ES:** ↑ insulin sensitivity

HIIE: high-intensity interval exercise. **SIE:** Sprint interval exercise. **WM:** work-matched. **ES:** extended sprint. **CMIE:** continuous moderate-intensity exercise. **NC:** no change compared to baseline or control. ↑ significant increase compared to baseline or control. ↓ significant decrease compared to baseline or control. **Young:** participants 18-40 years old. **Middle-aged:** 40-65 years old. **Older**: >65 years old. **T2DM:** type 2 diabetes mellitus. **MAGE:** mean amplitude of glycaemic excursions. **SD:** standard deviation of glycaemia. **Insulin clamp:** hyperinsulinaemic-euglycaemic clamp. **OGTT:** oral glucose tolerance test.

2.4.5 Post-exercise enhancement of insulin sensitivity.

It is generally conceded that training induced improvements in glycemic control lead to improved insulin action in part through the upregulation of key skeletal muscle glucose homeostasis regulatory proteins such as Akt1/2, AS160, AMPK, hexokinase 2, and importantly GLUT4 (179, 356). Improved insulin action may also occur through exercise-induced mitochondrial biogenesis and improved mitochondrial function in addition to the upregulation of antioxidant defenses that lead to improved redox homeostasis (179, 358). In contrast to regular exercise, the transient enhancement of insulin sensitivity in the hours after acute exercise appear to occur independent of modifications to the insulin receptor, IRS1/2, PI3K, Akt and/or GSK3 α/β proteins (127, 137, 355). It has been proposed that AS160 and TBC1D1, which converges downstream of insulin and contraction mediated glucose uptake signalling pathways, may play a role in enhanced post-exercise insulin action (62, 426). AS160 is a GTPase-activating protein for Rab family small G proteins which is now considered essential for insulin stimulated GLUT4 translocation and glucose uptake (64, 107, 194, 382, 419, 426). Insulin

stimulated Akt phosphorylation of AS160 promotes inactivation of the GDP bound form of the Rab proteins resulting in accumulation of the GTP bound active form, signalling GLUT4 vesicle movement and plasma membrane fusion (107, 374).

Numerous studies, although not all (64, 194), have reported increased phosphorylation of AS160 in the hours (up to 27 hours) after a single session of exercise in both animals and humans (135, 382, 425, 426, 441). The acute increase in AS160 phosphorylation after exercise is impaired in obese individuals and individuals with type 2 diabetes (401). In most cases, prior exercise enhances the insulin stimulated AS160 phosphorylation response coinciding with increased insulin sensitivity in humans and rodents (64, 135, 194, 426, 441). Combined with findings of impaired glucose kinetics in AS160 knockout mice (235, 453), it is evident that AS160 plays an important role in glucose homeostasis.

To date, the majority of human studies have used the non-site-specific PAS160 antibody which primarily detects AS160 phosphorylation on residue Thr642, and to a lesser extent Ser588 (139, 374). Recent research has however uncovered a potential phosphorylation site-specific role for AS160 (62, 139, 374, 427). Of the nine confirmed phosphorylation sites (i.e. Ser318, Ser341, Thr568, Ser570, Ser588, Thr642, Ser666, Ser704, and Ser751), phosphorylation on AS160 residues Ser588 and Ser751 appear to respond to skeletal muscle contraction, Ser318 responds to physiological levels of insulin, and Ser341, Thr642, Ser704 respond to both insulin and skeletal muscle contraction (427).

2.4.6 Exercise-intensity and AS160 signalling.

The most comprehensive investigation of exercise-intensity on AS160 phosphorylation was conducted by Treebak, et al. (425), however the effects on insulin stimulated AS160 and insulin sensitivity were not measured and the non-site-specific PAS160 antibody was used. Nevertheless, findings indicated that 1 minute, 10 minutes, and 30 minutes of continuous moderate-intensity cycling (~67% VO_{2peak}) had minimal impact on post-exercise PAS160 phosphorylation, whereas 60 minutes and 90 minutes of exercise at the same moderate-intensity increased PAS160 phosphorylation. Although a 2-minute cycle test at 110% of peak work rate and a 30 second Wingate test had minimal impact on PAS160 phosphorylation, 20 minutes of high-intensity cycling at 80% VO_{2peak} attenuated

PAS160 phosphorylation immediately after exercise (425). High-intensity resistance exercise also decreases IRS-1 tyrosine, PAS160, and Akt^{Ser473} phosphorylation immediately after exercise and impairs whole-body insulin sensitivity (188). Others have reported that 40 minutes of moderate-intensity exercise (70% VO_{2max}), but not low-intensity exercise (50% VO_{2max}), increases PAS160 phosphorylation immediately after, and 150 minutes after exercise in humans (401), although insulin action and glucose uptake was not measured. In summary, it appears that exercise-induced PAS160 phosphorylation occurs in an exercise-intensity and exercise-duration dependent manner, however further research is required to explore the role of exercise-intensity on site-specific AS160 phosphorylation.

Karstoft, et al. (211) recently reported that 4 months of walking exercise, consisting of 60 minute sessions of alternating between 3 min intervals of fast (≥70% of VO_{2peak}) and slow (~40% of VO_{2peak}) walking, 5 times per week, significantly improved glycaemic control, insulin sensitivity, and insulin stimulated $AS160^{Thr642}$ phosphorylation in individuals with type 2 diabetes. Importantly, training that incorporated only continuous walking of moderate-intensity (≥55% of walking VO_{2peak}) had minimal effect on these parameters despite being matched for energy expenditure and mean training-intensity. Thus, intermittent exercise may play an important role in exercised induced AS160 phosphorylation and glycaemic control, however research has yet to confirm these findings with acute HIIE.

2.5 Positive and negative regulation of glycaemic control by ROS.

Evidence provided so far supports acute exercise as an important modulator of oxidative stress, ROS, SAPK signalling, and glycaemic control. However, the interaction between these factors is not well understood. The following section discusses glycaemic control in the framework of exercise-induced oxidative stress, ROS and SAPK signalling.

2.5.1 Impaired glycaemic control.

Under normal physiological conditions, glucose and insulin rise substantially after nutrient ingestion (e.g. a meal), peak approximately 1 hour postprandial, and gradually return to baseline levels approximately 3-4 hours postprandial (31, 224) (Figure 2.6). Physical inactivity and obesity are associated with insulin resistance, largely due to impaired metabolism and subsequent accumulation of intracellular fat content in skeletal muscle, liver, adipose tissue, and pancreatic islets (122, 129, 350, 364). Increased resistance to insulin results in elevated postprandial glycaemia, which initially induces a compensatory response involving enhancement of β-cell function and increased β-cell mass (124). This compensatory response elevates basal and postprandial insulin secretion to levels above those measured in individuals that have normal glucose tolerance or T2DM (Figure 2.6). Eventually, compensatory mechanisms fail and chronic exposure of β-cells to abnormally high glucose and fatty acid concentrations leads to the loss of β-cell mass and function (58, 122, 129, 360). The resulting impairment in glucose disappearance, a condition involving a combination of defects in insulin secretion, insulin action, and glucose effectiveness (31, 36), leads to elevated basal and postprandial hyperglycaemia. Hyperglycaemia elicits systemic inflammation and oxidative stress which activate signalling pathways that perpetuate insulin resistance and contribute to the many health complications associated with type 2 diabetes (31, 473).

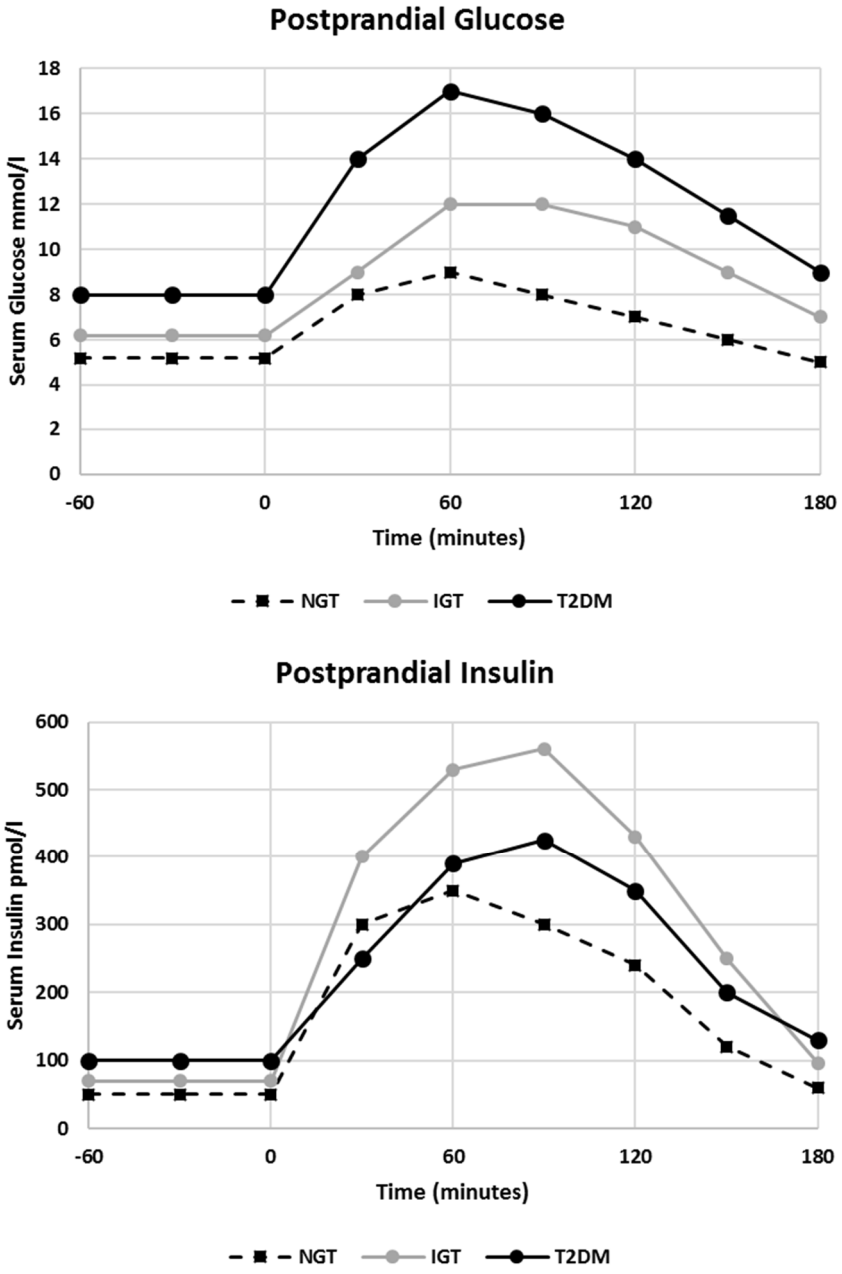

Figure 2.6: Postprandial serum glucose and insulin response to meal ingestion. **NGT:** normal glucose tolerance. **IGT:** impaired glucose tolerance. **T2DM:** individuals with type 2 diabetes. Hypothetical data adapted from Knudsen, et al. (224).

2.5.2 Physical inactivity, excess nutrient intake, and oxidative stress.

Chronic physical inactivity and over-nutrition are associated with elevated systemic oxidative stress and the development of lifestyle disease in part through mitochondrial dysfunction (122). Metabolism of carbohydrate and lipids initiates the transfer of electrons from reducing equivalents (i.e. NADH, $FADH_2$) into the mitochondrial electron transport system (ETS) (122). Increased electron flow

through the ETS promotes the pumping of protons outside the mitochondrial membrane creating a membrane potential. This membrane potential drives ATP synthase rephosphorylation of ADP to ATP, a process known as oxidative phosphorylation. The rate of oxidative phosphorylation relies primarily on energy demand rather than energy supply (122). In the absence of energy demand, for example physical inactivity, increased energy supply results in increased substrate metabolism, increased electron flow and proton transfer to the outer membrane. This leads to increased 'back pressure' of the mitochondrial membrane potential (102). Uncoupling proteins reduce the membrane potential through the uncoupling of ATP respiration to produce heat (11). When the membrane potential exceeds uncoupling capacity, electrons leak through complexes I and III reacting with O_2 to form the free radical O_2^- (10, 312). MnSOD catalyses O_2^- to H_2O_2. Providing there is sufficient antioxidant activity, H_2O_2 is further reduced to H_2O by antioxidants such as GSH and/or catalase (26). In pathological conditions in which antioxidant defence is insufficient, H_2O_2 can accumulate in the mitochondrial matrix and intermembrane space, or diffuse outside the permeable mitochondrial outer membrane (72). Excess ROS production results in oxidative stress and the signalling events leading to insulin resistance and chronic metabolic disease (473). This proposed mechanism for physical inactivity and excess nutrient intake induced chronic disease is supported by reports that mitochondrial specific antioxidants, which attenuate mitochondrial ROS production, reverses high-fat diet-induced insulin resistance in rodents (10).

Elevated basal and/or postprandial hyperglycaemia elicited through excess nutrient intake, physical inactivity, and insulin resistance, also increases oxidative stress through the formation of advanced glycation end products (AGEs) (38). Activation of the AGE receptor stimulates ROS production through NADPH oxidase (456), opening of the mitochondrial permeability transition pore (88), and through suppression of enzymatic antioxidant defences (37, 234, 475, 476). Hyperglycaemia therefore has the potential to elicit a potentially deleterious redox environment conducive to insulin resistance.

In humans, numerous studies have reported increased biomarkers of systemic oxidative stress for up to 4 hours after the ingestion of pure carbohydrate (286,

289), fat, and protein meals (288), and mixed macronutrient meals high in fat (59, 136, 434, 435), high in carbohydrate (414), and high fat liquid meals (43, 272). Larger meals and meals higher in lipid content elicit greater postprandial oxidative stress (45, 121). This has led to many studies researching the effects of high-fat meal ingestion on postprandial oxidative stress (47, 59, 136, 434, 435), however this does not represent the current Australian and New Zealand suggested dietary targets of approximately 45-65% carbohydrate, 20-35% fat and 15-25% protein (89). The effects of physiological meals on postprandial oxidative stress which more closely reflect recommended dietary guidelines have yet to be investigated.

A single bout of low to moderate-intensity exercise in healthy males can attenuate the postprandial oxidative stress response to a meal ingested 2 hours before exercise (272) and 24 hours after exercise (414), in part through improved glucose and triglyceride processing and clearance, and increased antioxidant activity (59). Acute high-intensity exercise may also attenuate postprandial oxidative stress (136, 435). However, findings from research investigating HIIE are inconsistent (59) and research is yet to investigate overweight, insulin resistant, and physically inactive populations.

2.5.3 Negative regulation of insulin signalling by ROS.

Sustained activation of redox-sensitive SAPK signalling pathways leads to impaired insulin signalling via serine and threonine phosphorylation of IRS 1 and 2 (86), summarised in Figure 2.7. Sustained IRS-1 serine phosphorylation at residues Ser307, Ser312, Ser616, and Ser1101 (human sequencing), impairs PI3K activity and downstream insulin signalling through attenuated tyrosine phosphorylation and IRS proteasomal degradation and subcellular re-localization (2, 3, 15, 34, 95, 100, 164-166, 244, 294, 459). The prevention of IRS-1 degradation through the inhibition of ROS and SAPK signalling restores insulin signalling and insulin-stimulated glucose uptake (138, 336, 398, 421). Surprisingly, IRS serine phosphorylation may also be necessary for insulin stimulated propagation of the insulin signalling cascade and glucose uptake (92), however reports are contradictory (149, 164, 459) and depend largely on the specific serine residue measured (457). Early phosphorylation of IRS-1 Ser307 and Ser323 after insulin stimulation is accompanied by enhanced tyrosine

phosphorylation and downstream insulin signalling, whereas sustained and maximal phosphorylation coincides with attenuation of the insulin signalling pathway and glucose uptake (457). Previous studies have also reported that hyperinsulinemia initiates a negative feedback loop that inhibits insulin signalling and glucose uptake in part through SAPK induced IRS serine phosphorylation (164, 238, 365, 459, 480).

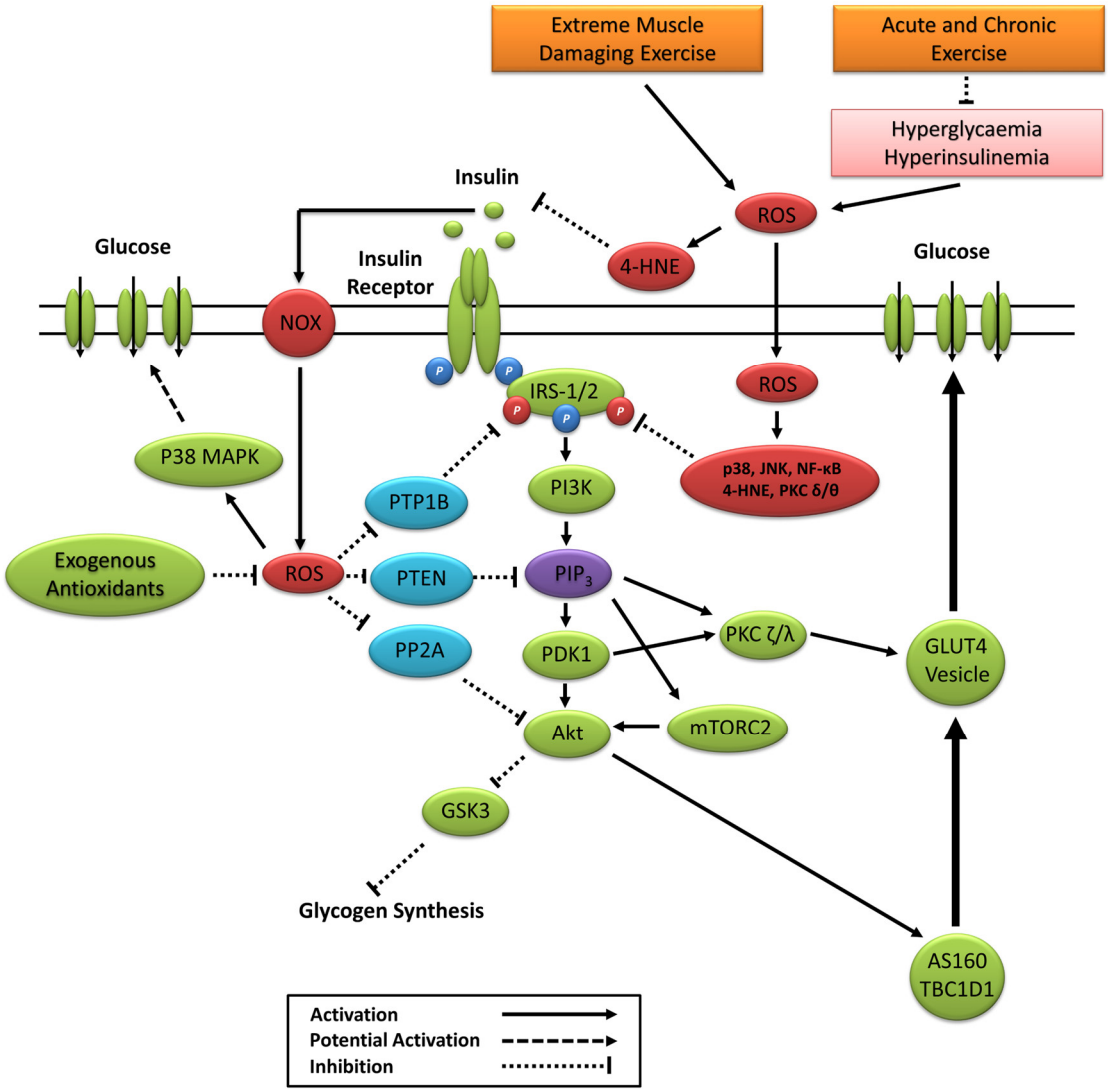

Figure 2.7: Primary ROS signalling pathways involved in positive and negative regulation of insulin signalling. Abbreviations: **4-HNE:** 4-hydroxynonenal; **Akt**: protein kinase B; **AS160**: Akt substrate of 160 kDa; **GSK3**: glycogen synthase kinase 3; **IRS-1/2**: insulin receptor substrate 1 and 2; **JNK**: c-Jun N-terminal kinases; **mTORC2**: mechanistic target of rapamycin complex 2; **NF-kB**: nuclear factor kappa-light-chain-enhancer of activated B cells; **NOX**: nicotinamide adenine dinucleotide phosphate-oxidase; **P38 MAPK**: p38 mitogen-activated protein kinases; **PDK1**: phosphoinositide-dependent kinase-1; **PI3K**: phosphatidylinositol-3 kinase; **PIP3**: phosphatidylinositol (3,4,5)-trisphosphate; **PKC**: protein kinase C; **PP2A**: protein phosphatase 2; **PTEN**: phosphatase and tensin homolog; **PTP1B**: protein-tyrosine phosphatase 1B; **RAC1**: ras-related C3 botulinum toxin substrate 1; **ROS**: reactive oxygen species. **TBC1D1**: TBC1 domain family member 1.

2.5.4 Positive regulation of insulin signalling by ROS.

The insulin receptor belongs to a subclass of the protein tyrosine kinase family. Positive regulation of the insulin signalling cascade is mediated in part through the oxidative inactivation of protein tyrosine phosphatases (PTP) which include PTP1B, phosphatase and tensin homolog (PTEN), and protein phosphatase 2 (PP2A) (Figure 2.7). Insulin induced inactivation of PTPs prevent the dephosphorylation of the insulin receptor (371), IRS-1 (152), and Akt proteins (415), and prevent the enzymatic degradation of PIP_3 (300), leading to increased glucose uptake. The PTP superfamily signature motif contains an invariantly low-p*Ka* catalytic cysteine residue making it highly susceptible to reversible oxidation by ROS (422). ROS inactivation of PTP activity plays a role in numerous cellular process including the regulation of cell proliferation, differentiation, survival, metabolism, and motility (422). Under basal conditions antioxidant defences including catalase and peroxiredoxin create a reduced intracellular redox environment prioritizing PTP activity. Increased PTP activity suppresses kinase activity and maintains a dephosphorylated state of the IR, IRS-1, and inhibition of the PI3K/Akt signalling pathway (300, 474). The binding of insulin to the insulin receptor signals a burst of endogenous superoxide production which is reduced to hydrogen peroxide creating a local oxidative redox environment (74, 257, 386). This oxidative redox environment favours the oxidation of catalytic cysteine to sulphenic acid, suppressing PTP activity and enhancing kinase activity and propagation of the insulin signalling cascade (122).

Insulin can elicit ROS production through enzymatic activation of NADPH oxidases (32, 74, 257, 386). Furthermore, insulin induced receptor tyrosine phosphorylation inactivates the endogenous membrane-associated antioxidant peroxiredoxin I, allowing for increased ROS production (470). Mahadev, et al. (257) reported that NADPH oxidase induced H_2O_2 enhances insulin signalling via oxidative inhibition of PTPs. Furthermore, palmitate induced insulin resistance in rat skeletal muscle occurs through increased activity of PTPs via JNK and NF-κB (287), which is reversed 16 hours after acute exercise in rats (362). Loh, et al. (253) revealed that the elevated H_2O_2 response to insulin in

GPx1$^{-/-}$ mouse embryo fibroblasts coincided with elevated PI3K/Akt signalling which can be suppressed by pre-treating cells with ebselen, an NADPH oxidase inhibitor, or the antioxidant N-acetylcysteine. Subsequent experiments revealed that elevated H_2O_2 in GPx1$^{-/-}$ mice increased PI3K/Akt signalling and glucose uptake through decreased PTP activity which was attenuated by the ingestion of n-acetylcysteine (253). Thus, redox mediated PTP activity appears to play a critical role in both positive and negative regulation of insulin signalling and glucose uptake.

2.5.5 Exercise-induced ROS, SAPK signalling and glycaemic control.

ROS are readily induced through contraction of skeletal muscle and activate redox-sensitive SAPK signalling pathways implicated in glucose metabolism (25, 34, 308, 321, 397, 420). Skeletal muscle SAPK signalling has therefore emerged as a potential candidate for the post-exercise enhancement of insulin sensitivity (253, 420). Loh, et al. (253) reported that elevated exercise-induced ROS in GPx1 knockout mice coincided with increased phosphorylation of Akt^{Ser473} and AS160^{Thr642}, and enhanced insulin stimulated glucose uptake 60 mins after a single session of treadmill exercise. This beneficial effect on insulin sensitivity was reversed with n-acetylcysteine supplementation, highlighting redox signalling as not only an important regulator of basal insulin signalling and glucose uptake, but also post-exercise enhancement of insulin sensitivity. Importantly, GPx1 knockout mice showed similar improvements in insulin sensitivity when measured immediately after exercise, supporting a growing consensus that the effects of post exercise-induced ROS on glycaemic control are temporal (324, 362).

One of the first studies to indicate a regulatory role of redox signalling in exercise-induced enhancement of insulin sensitivity in humans was conducted by Ristow, et al. (358). It was reported that vitamin C and E supplementation in humans attenuated the 4-week training induced improvements in insulin sensitivity and gene expression of PGC-1α/β, SOD, GPx1, and catalase (358). Not all studies in humans and rodents have reported impaired exercise-induced improvements in insulin protein signalling and insulin sensitivity with antioxidant supplementation (182, 335, 479). Contradictory findings likely stem from

variations in the type of antioxidant compound/s used, the dose used, the timing of supplementation, and the often non-specific and/or ineffective action of antioxidant supplementation for ROS inhibition in humans (170, 262, 270, 271, 302).

Enhanced glucose uptake approximately 4.5 hours after one-legged knee extensor exercise in humans is reported to coincide with greater basal and insulin stimulated p38 MAPK phosphorylation (420), highlighting SAPK signalling as a potential moderator of post-exercise glucose metabolism. Trewin, et al. (429) reported that n-acetylcysteine infusion attenuated whole-body insulin sensitivity approximately 5 hours after exercise. Phosphorylation of p38 MAPK was lower immediately after exercise with n-acetylcysteine infusion, however phosphorylation was not significantly different to baseline or the placebo after insulin stimulation. The null findings for p38 MAPK phosphorylation may, however, be due to the timing of post-exercise biopsies, the relatively small effect of n-acetylcysteine on insulin sensitivity (~6% reduction), and that n-acetylcysteine infusion was only infused during exercise and not during exercise recovery or the insulin clamp (429).

Berdichevsky, et al. (34) reported similar JNK phosphorylation in C2C12 myoblasts and L6 myotubes treated with chronic oxidative stress (1 μM of hydrogen peroxide for 48 hours) and acute oxidative stress conditions (500 μM of hydrogen peroxide for 3 hours). Interestingly, chronic oxidative stress decreased insulin induced Akt^{Ser473} phosphorylation, whereas acute oxidative stress enhanced insulin stimulated Akt^{Ser473} and GSK3-α/β phosphorylation. Furthermore, acute oxidative stress exposure in insulin resistant muscle cells rescues insulin-stimulated glucose uptake through increased IRS1 protein abundance, increased phosphorylation of JNK, Akt^{Ser473}, Akt^{Thr308} and GSK3-α/β, and decreased IRS-1^{Ser307} phosphorylation (34). In contrast, Ropelle, et al. (362) reported that a single bout of exercise in male rats reverses diet-induced insulin resistance 16 hours later via attenuation of JNK, NF-κB, and IRS-1^{Ser307} signalling. It is possible that acute exercise enhances insulin signal transduction through the transient and immediate increase in ROS and SAPK signalling, which also leads to a delayed increase in antioxidant activity and subsequent

attenuation of chronic oxidative stress and sustained SAPK signalling pathways associated with insulin resistance. Certainly, SOD protein content, SOD activity, and total antioxidant status increase and/or remain elevated for up to 16-24 hours after exercise (109, 184, 435), whereas lipid-induced postprandial oxidative stress is attenuated (136).

A summary of glycaemic control protein signalling pathways and the potential role of ROS and SAPK signalling are presented in Figure 2.8. Evidence provided so far supports a role for ROS and SAPK signalling in glycaemic control, however the role of exercise-induced ROS and SAPK signalling are less clear and warrant further investigation (Table 2.5).

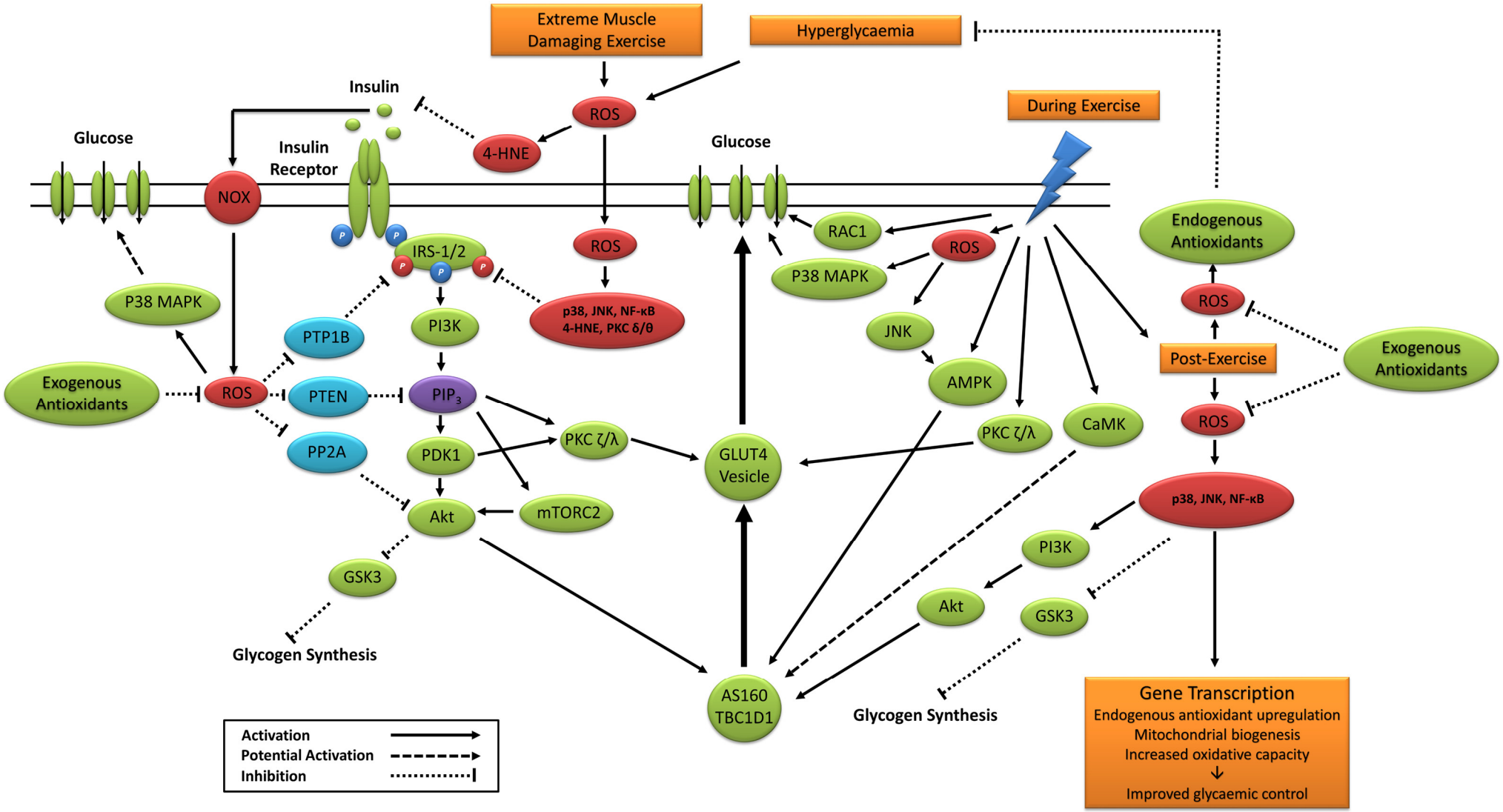

Figure 2.8: Primary signalling pathways involved in contraction and insulin stimulated glucose uptake and the potential role of ROS. Abbreviations: **4-HNE:** 4-hydroxynonenal; Akt: protein kinase B; **AMPK:** 5' adenosine monophosphate-activated protein kinase; **AS160:** Akt substrate of 160 kDa; **CaMK:** ca2+/calmodulin-dependent protein kinase; **GLP-1:** glucagon-like peptide-1; **GLUT4:** glucose transporter 4; **GSK3:** glycogen synthase kinase 3; **IL-6:** interleukin 6; **IRS-1/2:** insulin receptor substrate 1 and 2; **JNK:** c-Jun N-terminal kinases; **mTORC2:** mechanistic target of rapamycin complex 2; **NF-kB:** nuclear factor kappa-light-chain-enhancer of activated B cells; **NOX:** nicotinamide adenine dinucleotide phosphate-oxidase; **P38 MAPK:** p38 mitogen-activated protein kinases; **PDK1:** phosphoinositide-dependent kinase-1; **PI3K:** phosphatidylinositol-3 kinase; **PIP3**: phosphatidylinositol (3,4,5)-trisphosphate; **PKC:** protein kinase C; **PP2A:** protein phosphatase 2**; PTEN**: phosphatase and tensin homolog; **PTP1B:** protein-tyrosine phosphatase 1B; **RAC1:** ras-related C3 botulinum toxin substrate 1; **ROS:** reactive oxygen species. **TBC1D1**: TBC1 domain family member 1.

Table 2.5: Summary of key findings from research investigating acute exercise, redox status and enhancement of glycaemic control.

Reference	Participants/ Animals/Cells*	Exercise stimulus	Redox manipulation	Time point/conditions	SAPK signalling	Glycaemic control
Berdichevsky, et al. (34)	Myocytes, myoblasts and/or myotubes	Acute oxidative stress (simulated exercise)	Chronic oxidative stress	Chronic oxidative stress	↑ p-JNK	↓ p-Akt^{Ser473,Thr308}. ↓ glucose uptake
			Acute oxidative stress	Acute oxidative stress	↑ p-JNK	↑ p-Akt^{Ser473,Thr308} and p-GSK3β. ↑ glucose uptake
				Insulin resistant cells	↑ p-JNK	↑ p-IRS-1^{Ser312}, ↓ IRS-1 and glucose uptake
				Insulin resistant cells + acute oxidative stress	Greater ↑ p-JNK	↓ p-IRS-1^{Ser312}. ↑ IRS-1 and glucose uptake.
Castorena, et al. (64)	Low-fat and high-fat diet fed rats.	Swimming exercise.		3h post-ex. + Ins.	**NC** p-JNK	**NC** Akt^{Ser473,Thr308}, IR$^{Tyr1162/1163}$, IRS-1-PI3K ↑ pAS160^{Thr642,Ser588} ↑ Insulin sensitivity Greater ↑ in insulin sensitivity and pAS160^{Thr642,Ser588} in low-fat diet fed rats
Geiger, et al. (138)	Male Wistar rats	In vitro contraction	p-p38 MAPK inhibition	3h post-contraction	↑ p-p38 MAPK	Not measured
				3h post-contraction. + P38 MAPK inhibition	**NC** p-p38 MAPK	Not measured
				Post-Ins.	Not measured	↑ glucose uptake
				3h post contraction + Ins.	Not measured	Greater ↑ glucose uptake
				3h post contraction + Ins. + p38 MAPK inhibition.	Not measured	Similar glucose uptake as previous condition
Loh, et al. (253)	10 Wild Type mice 9 GPx1−/− mice	Treadmill exercise	GPx1 −/− and N-acetylcysteine	Post-ex. + ins.		GPx1−/− mice: Similar insulin sensitivity.
				1h post-ex. + ins.		GPx1−/− mice: Greater ↑ p-Akt^{Ser473} and insulin sensitivity. N-acetylcysteine: **attenuated** insulin sensitivity.

Ropelle, et al. (362)	Male Wistar rats Control (n=6) Obese (n=8) Obese + Ex. (n=8)	Swimming exercise		16h post-ex. + Ins.	Compared to Control: Obese ↑ p-JNK. ↓ IκBα. Compared to Obese: Obese + Ex. ↓ p-JNK. ↑IκBα.	Compared to both Control and Obese + Ex.: Obese **attenuated** insulin sensitivity, PI3K, p-IRS-1/2, p-IR. ↑ PTP1B content/activity and p-IRS^{Ser312}.
Somwar, et al. (398)	Male Wistar rats	In vitro contraction	p-p38 MAPK inhibition	Post-Ins. Post-Ins. + p38 MAPK inhibition Post-contraction. Post-contraction + p38 MAPK inhibition	↑ p-p38 MAPK and activity **Attenuated** p38 MAPK activity ↑ p-p38 MAPK and activity **Attenuated** p38 MAPK activity	Not measured **Attenuated** glucose uptake Not measured **Attenuated** glucose uptake
Thong, et al. (420)	7 Young Males Active Healthy	60 mins of one-legged knee extension.		3h post-ex. 3h post-ex. + 30min Ins. 3h post-ex + 100min Ins.	↑ p-p38 MAPK Greater ↑ p-p38 MAPK p-p38 MAPK similar to previous time point	Not measured ↑ insulin sensitivity compared to control leg. ↑ insulin sensitivity compared to control leg.
Trewin, et al. (429)	7 Young Adults Active Healthy	55 mins at 65% VO_{2peak} followed by 5 mins @ 85% VO_{2peak}	Crossover: N-acetylcysteine and Saline infusion	Post-ex. 3h post-ex. 3h post-ex. + 2h Ins.	NAC: ↓ p-p38 MAPK Both: **NC** p-p38 MAPK Both: **NC** p-p38 MAPK	Both: **NC** p-Akt^{Thr308,Ser473} and PAS160. Both: ↑ PAS160. **NC** p-Akt^{Thr308,Ser473}. Both: ↑ p-Akt^{Thr308,Ser473} and PAS160. NAC: ↑ insulin sensitivity

Ins.: insulin stimulation. **NC:** no change compared to baseline or control. ↑ significant increase compared to baseline or control. ↓ significant decrease compared to baseline or control. *Where appropriate sample sizes for animal research are reported.

2.5.6 Potential mechanisms linking SAPK signalling and enhancement of glycaemic control.

Modulation of glycogen synthesis by oxidative stress-induced SAPK signalling is reported to play a role in glucose metabolism (34, 98, 100, 420). Transient stimulation of C2C12 myoblasts with hydrogen peroxide increases JNK, Akt and GSK-3 α/β phosphorylation (34), suggesting acute exercise-induced ROS may increase glycogen synthesis and skeletal muscle glucose uptake. Likewise, post-exercise enhancement of insulin stimulated p38 MAPK phosphorylation is associated with post-exercise glycogen depletion (420). Chan et al. (71) established that low intramuscular glycogen was associated with greater phosphorylation of nuclear p38 MAPK after 60 minutes of cycle exercise. In contrast, insulin stimulation of rat skeletal muscle exposed to 1 hour of H_2O_2 (~90 μM) exhibits impaired insulin protein signalling, glycogen synthesis, and glucose uptake, despite increased p38 MAPK phosphorylation (100). Diamond-Stanic, et al. (98) reported similar findings and proposed that p38 MAPK and GSK-3 were unlikely to play a beneficial role in insulin stimulated glucose uptake.

Other potential pathways include JNK, NF-kB, and p38 MAPK stimulated secretion of the recently identified insulin sensitizing Interleukin-6 (IL-6) (61, 116, 321, 400). Carey, et al. (61) reported that IL-6 infusion increases insulin stimulated glucose uptake in humans. Furthermore, IL-6 treatment in L6 myotubes coincides with increased glucose uptake and GLUT4 translocation, likely through AMPK pathways independent of the canonical insulin signalling cascade (61). Importantly, IL-6 secretion is increased following muscular contraction, likely via activation of JNK, NF-kB and/or p38 MAPK (71, 440, 465). It has also been reported that p38 MAPK inhibiters, alongside expression of a dominant-negative p38 mutant, impairs insulin stimulated glucose uptake without reductions in GLUT4 translocation (397). Researchers concluded that p38 MAPK may exert its insulin sensitizing effect through increased activation of translocated GLUT4 (397), but this has yet to be confirmed in humans (132). Subcellular redistribution of phosphorylated JNK from the cytoplasm to the nucleus after exercise may also play an insulin sensitizing role (34), however

few studies exist and research is yet to investigate this mechanism in humans and with other SAPK proteins.

2.6 Summary, aims, and hypotheses.

This review of the literature has revealed an apparent redox paradox. Physical inactivity and excess adipose tissue is associated with elevated ROS production, systemic oxidative stress, and sustained activation of SAPK signalling pathways which involve JNK, NF-kB and p38 MAPK. If left unchecked, this chronic state of physiological stress leads to the development of insulin resistance and metabolic disease. Paradoxically, a single session of exercise transiently increases ROS, oxidative stress and SAPK signalling pathways, however this occurs prior to, or concomitant with, enhanced insulin sensitivity and metabolic adaptation.

Previous studies have shown that inhibition of exercise-induced ROS and oxidative stress via the administration of reducing compounds, such as antioxidant supplements, have in many cases attenuated exercise-induced improvements in insulin sensitivity, antioxidant defence upregulation, and markers of mitochondrial biogenesis. Likewise, increased exercise-induced oxidative stress, as found in antioxidant depleted or knockout mice, show increased insulin sensitivity and/or upregulation of markers of mitochondrial biogenesis. It is now apparent that exercise-induced redox-sensitive SAPK signalling is beneficial and a necessary requirement for adaptation to physiological stress.

Exercise consisting of short bouts of high-intensity exercise, interspersed with short active and/or passive recovery periods, is a popular exercise mode that elicits comparable, and in some cases greater improvements in cardiometabolic health compared to traditional CMIE. Although exercise-induce oxidative stress and antioxidant defence are reported to mediate many health benefits of regular exercise, the effects of acute HIIE and LV-HIIE/SIE on post-exercise changes in systemic oxidative stress and antioxidant defence are unclear and yet to be adequately investigated in humans. Skeletal muscle SAPK signalling is redox-sensitive, increases transiently after a single session of exercise, and reported to play a role in exercise-mediated adaptation and insulin protein signalling.

However, comparison of exercise-induced changes to redox status, SAPK signalling, and insulin protein signalling, between HIIE, SIE, and CMIE, has yet to be conducted. As such, the aims of Chapter 3 were to investigate and compare the acute exercise-induced plasma redox status, and skeletal muscle SAPK and insulin protein signalling response to SIE, HIIE, and CMIE, in young recreationally active adults. It was hypothesized that SIE and HIIE would elicit greater post-exercise systemic oxidative stress and antioxidant activity, and greater post-exercise skeletal muscle SAPK signalling, compared to CMIE. It was further hypothesized that elevated oxidative stress and SAPK signalling after exercise would coincide with enhanced insulin protein signalling.

To further explore the role of exercise-induced oxidative stress and SAPK signalling in metabolic health, an experiment was conducted in obese middle-aged males, whom are reported to exhibit greater basal oxidative stress, SAPK signalling, and insulin resistance. The aims of this experiment (Chapter 4) were to establish whether changes in insulin sensitivity after acute HIIE were aligned with changes in exercise-induced systemic redox status and skeletal muscle SAPK and insulin protein signalling. It was hypothesized that HIIE would significantly increase post-exercise plasma oxidative stress and antioxidant defence, and increase skeletal muscle SAPK and insulin signalling in obese middle-aged men. It was further hypothesized that exercise-induced oxidative stress and SAPK signalling would coincide with enhanced insulin sensitivity and insulin protein signalling.

Postprandial oxidative stress and impaired glycaemic control is associated with insulin resistance and metabolic disease. A single session of exercise can decrease postprandial oxidative stress and glycaemia, however the effects of LV-HIIE in overweight and physically inactive individuals are yet to be investigated. Furthermore, laboratory based methods for measuring insulin sensitivity and glycaemic control, such as the hyperinsulinaemic-euglycaemic clamp, may not accurately reflect glucose and insulin dynamics under physiological conditions and can underestimate functional improvements in glycaemic control. Previous research has indicated continuous glucose monitoring (CGM) to be a reliable and valid method for measuring 24-hour glycaemic status, glycaemic variability, and postprandial responses to meals

under free-living conditions. Research has yet to use CGM to investigate post-exercise glycaemic control after LV-HIIE that is not work-matched to CMIE. As such, the final experimental chapter (Chapter 5) investigated the effect of a single session of LV-HIIE and CMIE on postprandial oxidative stress and glycaemia, and 24-hour glycaemic control as measured by CGM in overweight and physically inactive adults. It was hypothesized that a single session of LV-HIIE would decrease postprandial oxidative stress and glycaemia, and improve 24-hour glycaemic control, to a greater extent than CMIE.

CHAPTER 3. THE EFFECT OF EXERCISE-INTENSITY ON PLASMA REDOX STATUS AND SKELETAL MUSCLE STRESS KINASE AND INSULIN PROTEIN SIGNALLING.

Data presented within this chapter has been adapted from:

Parker, L., Trewin, A., Levinger, I., Shaw, C. S., & Stepto, N. K. (2017). The effect of exercise-intensity on skeletal muscle stress kinase and insulin protein signaling. PLoS ONE, 12(2), e0171613.

Parker, L., Trewin, A., Levinger, I., Shaw, C. S., & Stepto, N. K. (2017). Exercise-intensity dependent alterations in plasma redox status do not reflect skeletal muscle redox-sensitive protein signaling. JSAMS.

3.0 General introduction.

We previously investigated the effect of increasing exercise-intensity, interspersed with short rest periods, on post-exercise plasma oxidative stress and endogenous antioxidant defence (323). These findings confirmed previous reports that higher-intensity exercise elicits greater ROS, as indicated by increased plasma biological antioxidant potential at 70%, 85%, and 100% of VO_{2max}. Oxidative stress measured via the reactive oxygen metabolite assay was not significantly elevated after exercise at any intensity, suggesting that antioxidant defence was sufficient to prevent exercise-induced oxidative stress. However only one biomarker of plasma oxidative stress was measured and only five minute bouts of exercise were investigated. Nevertheless, increased plasma antioxidant defence with higher-intensity exercise suggested a post-exercise redox homeostatic response for the prevention of excess oxidative damage to lipids, proteins and DNA. Previous research has reported that repeat stimulation of this redox response through regular exercise leads to the upregulation of endogenous antioxidant defence which helps protect against oxidative stress associated chronic lifestyle disease. Our findings highlighted acute high-intensity exercise as a potent stimulus for the transient induction of plasma antioxidant defence. However, further research is required to confirm these findings with other modes of high-intensity exercise, such as high-intensity interval-exercise (HIIE), and to measure multiple biomarkers of redox status.

HIIE is reported to provide a strong stimulus for exercise-induced skeletal muscle adaptation. Furthermore, low-volume sprint interval exercise (SIE), which consists of shorter duration all-out efforts, is often reported to provide similar adaptions to HIIE and traditional continuous moderate-intensity exercise (CMIE), despite consisting of less total work. However, the effects of HIIE and SIE on post-exercise redox status compared to CMIE is unclear. Furthermore, whether changes in systemic redox status with higher-intensity exercise coincide with changes in redox-sensitive SAPK signalling in skeletal muscle are unknown. Elucidating this exercise response is important as SAPK signalling is important for skeletal muscle adaptation and may affect muscle insulin protein signalling and glucose homeostasis. The aim of this chapter was to compare

the acute exercise-induced systemic redox status response and skeletal muscle SAPK and insulin protein signalling response between low-volume SIE, CMIE, and HIIE work-matched to CMIE, in young recreationally active adults.

3.1 Abstract.

Background. Redox status and stress-activated protein kinase (SAPK) signalling play an important role in glucose homeostasis and the physiological adaptation to exercise. However, the effects of acute high-intensity interval exercise (HIIE) and sprint interval exercise (SIE) on activation of these signalling pathways are unclear. **Methods.** Eight young and recreationally active adults performed a single cycling session of HIIE (5 x 4 minutes at 75% W_{max}), SIE (4 x 30 second Wingate sprints), and continuous moderate intensity exercise work-matched to HIIE (CMIE; 30 minutes at 50% of W_{max}), separated by a minimum of 1 week. Plasma redox status was measured immediately after exercise, 1 hour, 2 hours and 3 hours after exercise. Skeletal muscle SAPK and insulin protein signalling were measured immediately after exercise and 3 hours after exercise. **Results.** SIE elicited greater plasma catalase activity (SIE: ~58%; HIIE: ~21%; CMIE; ~-12%; $p < 0.05$) and skeletal muscle NF-κB p65 phosphorylation immediately after exercise (SIE: ~40%; HIIE: ~4%; CMIE; ~13%; $p < 0.05$) compared to HIIE and CMIE. Plasma hydrogen peroxide (~61%; $p < 0.05$) and skeletal muscle JNK (~42%; $p < 0.05$) and p38 MAPK (~171%; $p < 0.05$) phosphorylation increased, and plasma TBARS (~-17%; $p < 0.05$), SOD activity (~-40%; $p < 0.05$), and skeletal muscle Akt^{Ser473} phosphorylation (~-32%; $p < 0.05$) decreased, to a similar extent after all exercise protocols. $AS160^{Ser588}$ phosphorylation was similar to baseline three hours after SIE (~-12%; $p > 0.05$), remained lower 3 hours after HIIE (~-34%; $p < 0.05$), and decreased 3 hours after CMIE (~-33%; $p < 0.05$). **Conclusion.** Despite consisting of less total work than CMIE and HIIE, SIE proved to be an effective stimulus for the alteration of plasma redox homeostasis and activation of stress protein kinase signalling pathways linked to exercise-mediated adaptation of skeletal muscle. Furthermore, post-exercise $AS160^{Ser588}$ phosphorylation decreased in an exercise-intensity and post-exercise time-course dependent manner.

3.2 Introduction.

High-intensity interval-exercise (HIIE) and sprint-interval exercise (SIE) are reported to elicit comparable, and in some cases, greater improvements in measures of glycaemic control, oxidative stress, and mitochondrial biogenesis, compared to continuous moderate-intensity exercise (CMIE) (141, 251, 348, 418, 460). The mechanisms for improved skeletal muscle adaptation after HIIE and SIE are unclear, but may involve exercise-induced stress protein kinase signalling (142, 156, 181, 209).

Physical inactivity and excess adipose tissue can lead to the sustained activation of mitogen and stress-activated protein kinases (SAPK), in-part through increased mitochondrial electron leak and the subsequent production of reactive oxygen species (ROS) (122, 421). Important ROS sensitive SAPK proteins include c-Jun N-terminal kinases (JNK), p38 mitogen-activated protein kinases (p38 MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Sustained activation of these protein signalling pathways leads to impaired insulin sensitivity in part through serine phosphorylation of the insulin receptor substrate 1 (IRS-1), IRS-1 degradation and attenuation of distal insulin signalling proteins such as Akt substrate 160 (AS160) (22, 338, 421). Paradoxically, acute exercise also results in increased ROS production (120), albeit transiently and predominantly through NADPH oxidase superoxide anion production (369), which is reported to contribute to the transient activation of SAPK signalling in skeletal muscle (228). In contrast to the sustained activation of SAPK signalling, the transient activation following acute exercise coincides with greater AS160 phosphorylation post-exercise and improved insulin sensitivity (127, 324, 426). Furthermore, exercise-induced SAPK signalling is also linked to the activation of skeletal muscle transcription factors and coactivators that lead to skeletal muscle adaptation and long-term improvements in cardiometabolic health (228, 251, 346, 348).

Although HIIE and SIE training are reported to elicit equivalent and in some cases superior exercise-mediated cardiometabolic adaptations when compared to CMIE (141, 251, 348), the effects of acute HIIE and SIE on post-exercise skeletal muscle SAPK signalling are equivocal. For example, greater metabolic fluctuations induced through intermittent exercise are considered to

elicit greater post-exercise p38 MAPK phosphorylation (81). However, previous studies have reported similar exercise-induced p38 MAPK phosphorylation after acute work-matched HIIE, SIE, and continuous exercise (29, 79). The effects of low-volume SIE, compared to higher-volume HIIE work-matched to continuous exercise of moderate-intensity, on post-exercise skeletal muscle p38 MAPK phosphorylation are unknown. Furthermore, skeletal muscle JNK and NF-κB phosphorylation and post-exercise insulin protein signalling have yet to be explored after acute HIIE and SIE.

We compared the effects of a single session of HIIE, SIE, and CMIE work-matched to the HIIE, on systemic redox status and skeletal muscle SAPK and insulin signalling protein activation/phosphorylation. It was hypothesized that SIE and HIIE would elicit greater plasma oxidative stress, antioxidant capacity, and skeletal muscle SAPK and distal insulin protein signalling.

3.3 Methodology and procedures.

3.3.1 Participants.

Participants. Eight recreationally active adults, 6 males and 2 females, volunteered to participate in this randomized cross-over study. Participant characteristics are reported in Table 1. Exclusion criteria for participation included smoking, musculoskeletal or other conditions that prevent daily activity, symptomatic or uncontrolled metabolic or cardiovascular disease, and females taking oral contraception. To minimize the effect of hormonal fluctuations on outcome measures, females were tested in the early follicular phase of the menstrual cycle (2-7 days after the onset of menses). Verbal and written explanations about the study were provided prior to obtaining written informed consent. This study was approved by and carried out in accordance with the Victoria University Human Research Ethics Committee for experiments involving humans.

Table 3.1: Descriptive characteristics of participants.

Variable	N = 8
Participants	6 males and 2 females
Age (years)	25 ± 2
Height (cm)	179.3 ± 2.9
Weight	79.4 ± 2.1
BMI ($kg \cdot m^{-2}$)	25 ± 1
W_{max} during GXT (W)	327 ± 25
Max heart rate during GXT (BPM)	183 ± 4
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	48.4 ±4 .0

Values are mean ± SEM.

Participants were asked to abstain from physical activity (~72 hours), alcohol and caffeine consumption (~24 hours) prior to each trial. Twenty-four hours before their first trial volunteers were asked to consume their habitual diet which was recorded in a diet diary and replicated in their subsequent trials. Participants completed a screening session prior to completing the three different exercise protocols in a randomized crossover fashion, separated by a minimum of 1 week for males and ~4 weeks for females (Figure 3.1).

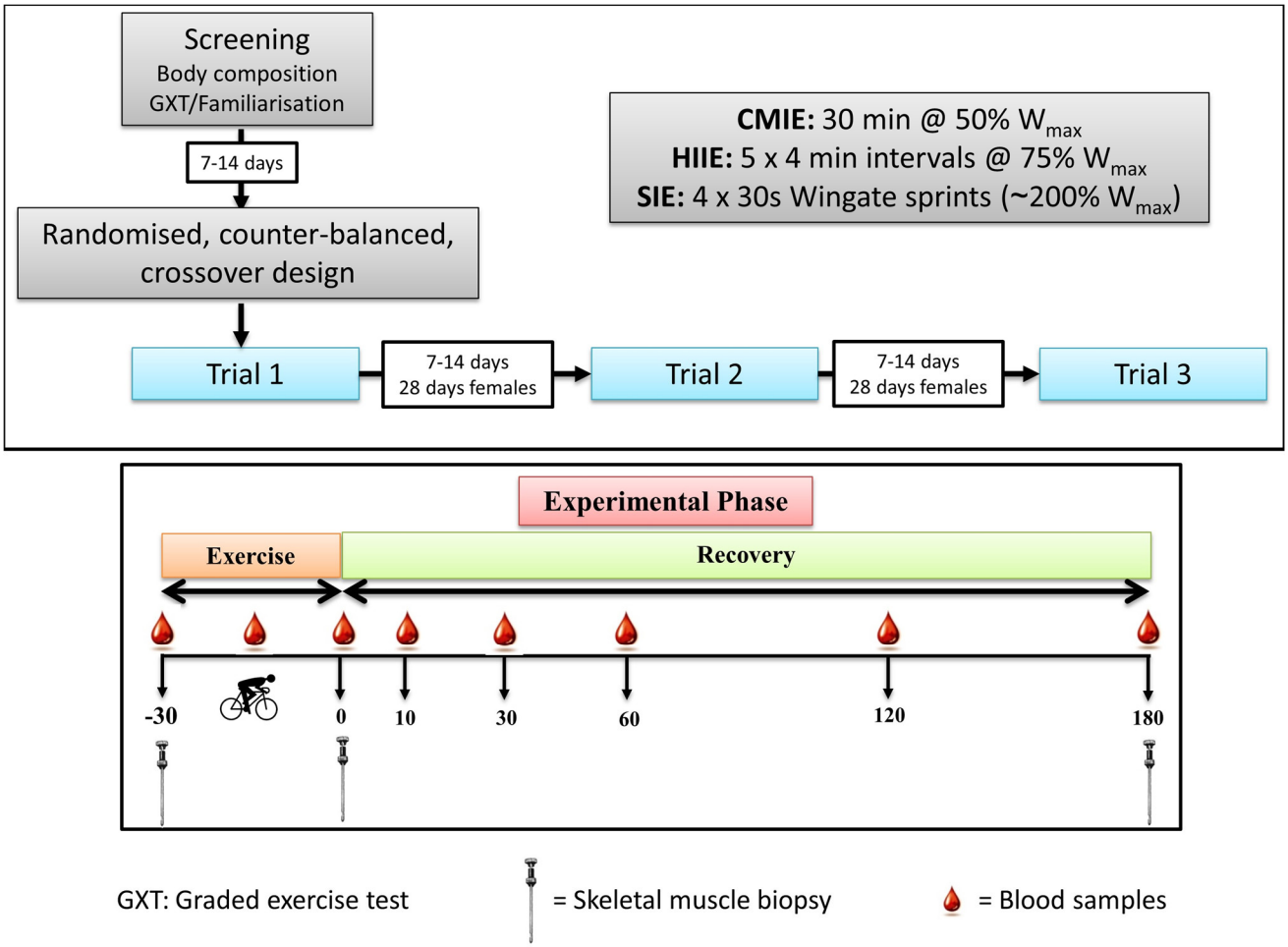

Figure 3.1: Schematic overview of study 1 research methodology. After initial screening and determination of W_{max} and VO_{2peak}, participants underwent three exercise sessions, separated by 7-14 days (~28 days for females), in a randomized crossover fashion. Venous blood and skeletal muscle samples were taken at time-points indicated in the figure. CMIE: continuous moderate-intensity exercise. HIIE: high-intensity interval exercise. SIE: sprint interval exercise. GXT: graded exercise test.

3.3.2 Screening and preliminary testing.

Participants were screened via a medical history and risk assessment questionnaire. Eligible participants underwent anthropometric measurement (height and weight) and completed a graded exercise test (GXT) on a cycle ergometer (Velotron, USA) to measure peak aerobic capacity (VO_{2peak}) and maximal power output (W_{max}). The GXT protocol consisted of 1-minute cycling stages at 50 watts which increased by 25 watts every minute until participants were unable to maintain a cycling cadence of 60 RPM or greater. Expired gases were collected and analysed via an indirect calorimetry system (Moxus Modular VO_2 System, USA). The W_{max} obtained during the GXT was used to calculate the workload for the three exercise protocols.

3.3.3 Experimental phase.

On three separate occasions participants reported to the laboratory in the morning after an overnight fast. A resting muscle biopsy and venous blood sample were taken prior to participants undergoing their randomized exercise protocol (SIE, HIIE or CMIE). Immediately following the acute session of exercise, a muscle biopsy and venous blood sample were taken, and participants rested on a bed for three hours. A third muscle biopsy was taken 3 hours after exercise and venous blood samples were taken in the middle of the exercise session, immediately after exercise, and 10 minutes, 30 minutes, 1 hour, 2 hours and 3 hours after exercise.

3.3.4 Exercise protocols.

All exercise sessions were performed on a Velotron cycle ergometer. The SIE protocol consisted of 4 x 30 second all-out (Wingate) cycling sprints, interspersed with 4.5-minute passive recovery periods. Pedalling resistance for the SIE was determined as a torque factor relative to body mass which was optimized during the familiarization session. The HIIE protocol consisted of 5 x 4-minute cycling bouts at 75% of W_{max} (~77% of VO_{2peak}), interspersed with 1-

minute passive recovery periods. The CMIE protocol consisted of continuous cycling for 30 minutes at 50% of W_{max} (~54% of VO_{2peak}), equating to the same total work performed (294 ± 23 kJ) in the HIIE protocol.

3.3.5 Skeletal muscle and blood sampling.

Muscle samples were obtained from the vastus lateralis under local anaesthesia (Xylocaine 1%, Astra Zeneca, Australia) utilizing a Bergström needle with suction (113). The samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Venous blood was collected from an antecubital vein via an intravenous cannula and analysed immediately for blood glucose and lactate using an automated analysis system (YSI 2300 STAT Plus™ Glucose & Lactate Analyser). Additional venous blood was collected in collection tubes containing ethylenediaminetetraacetic acid (EDTA), separated into plasma by centrifugation (10 min at 3500 rpm, 4°C), then aliquoted and stored at -80°C until analysed.

3.3.6 Plasma redox status analysis.

Plasma thiobarbituric acid reactive substances (TBARS; Cayman Chemical, Ann Arbor, MI, USA), Catalase activity (Cayman Chemical, Ann Arbor, MI, USA), Superoxide Dismutase (SOD) activity (Cayman Chemical, Ann Arbor, MI, USA) and Hydrogen Peroxide (Amplex UltraRed assay; Molecular Probes, Eugene, Oregon, USA) were analysed by a spectrophotometer (xMark microplate spectrophotometer, Bio-Rad Laboratories, Mississauga, ON, Canada) in duplicate as per the manufacturer's instructions. One unit of catalase activity is defined as the amount of enzyme required to cause the formation of 1.0 nmol of formaldehyde per minute at 25°C. One unit of SOD activity is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Intra-assay coefficients of variation were determined and averaged from each duplicate for all participants and resulted in a coefficient of variation of 2%, 5%, 4% and 3% for TBARS, SOD, catalase and hydrogen peroxide, respectively. Inter-assay coefficients of variation for assay standards between each 96 well plate were averaged and resulted in a coefficient of variation of 1%, 3%, 4%, 1%, for TBARS, SOD, catalase and hydrogen peroxide, respectively.

3.3.7 Skeletal muscle protein analysis.

To avoid the potential loss of total cellular protein that can occur with centrifugation (292, 296), phosphorylation and abundance of specific proteins in whole muscle lysate were determined with all constituents present (i.e. no centrifugation). Whole muscle lysate was analysed as previously reported (324). In brief, thirty cryosections of skeletal muscle (20 µm) were homogenized in buffer (0.125M TRIS-HCL [pH 6.8], 4% SDS, 10% Glycerol, 10mM EGTA, 0.1M DTT, and with 0.1% v/v protease and phosphatase inhibitor cocktail [#P8340 and #P5726, Sigma Aldrich]). Total protein content of muscle lysate was determined using the commercially available Red 660 Protein Assay kit with SDS neutralizer as per the manufacturer's instructions (Red 660, G-Biosciences, St. Louis, MO, USA). Eight µg of protein was prepared in 3 µl of Bromophenol blue (1%), heated for 5 minutes at 95°C and separated by 7.5% Criterion™ TGX™ Pre-Cast Gels. The separated proteins were transferred to a polyvinylidene difluoride membrane and blocked with Tris-Buffered Saline-Tween (TBST) and 5% skim milk for 1 hour. Membranes were washed (4 x 5 minutes) with TBST and incubated at 4°C overnight with the following primary antibodies: phospho-SAPK/JNK (Thr183/Tyr185; CST #9251), SAPK/JNK (CST #9252), phospho-p38 MAPK (Thr180/Tyr182; CST #9211), p38 MAPK (CST #9212), phospho-NF-κB p65 (Ser536; CST #3033), NF-κB p65 (CST #8242), IκBα (CST #4814), phospho-IRS-1 (Ser307 in human; CST #2384), phospho-AS160 (Ser588; CST #8730), AS160 (CST #2447), phospho-Akt (Ser473; CST #9271), Akt (#9272), and IRS-1 (Millipore, 06-248). After incubation, membranes were washed with TBST and incubated for 1 hour at room temperature with appropriate dilutions of horseradish peroxidase conjugated secondary antibody. Membranes were re-washed and incubated in SuperSignal West Femto Maximum Sensitivity substrate for 5 minutes prior to imaging. After imaging, membranes were stained via a modified Coomassie staining protocol (324). All densitometry values are expressed relative to a pooled internal standard and normalized to the total protein content of each lane obtained from the modified Coomassie staining protocol. Where appropriate, phosphorylated proteins are expressed relative to specific total protein content.

3.3.8 Statistical analysis and sample size calculation.

Data were checked for normality and analysed using Predictive Analytics Software (PASW v20, SPSS Inc., Chicago, WI, USA). Comparisons of multiple means were examined using a repeated measures analysis of variance (exercise protocol x time point). Post hoc analysis of significant interaction and main effects were performed using Fisher's protected LSD test. All data are reported as mean ± standard error of mean (SEM) and statistical analysis conducted at the 95% level of significance ($p \leq 0.05$). Trends were reported when p-values were greater than 0.05 and less than 0.1.

A priori calculations (G*Power 3.1.9) indicated that with an alpha set at 0.05 and power at 0.8, a sample size of six in each group was required to detect a significant ($p < 0.05$) increase in phosphorylated p38 MAPK immediately after exercise. This calculation was based on a previously reported effect size f of 1.14 (90 ± 70% increase in phosphorylation; n = 10) (29). Thus, the study was adequately powered for the main outcome measure of post-exercise p38 MAPK phosphorylation.

3.4 Results.

3.4.1 Blood glucose and lactate.

Significant interaction effects ($p < 0.05$) were detected for blood glucose and lactate ($p < 0.05$). Post-hoc analysis revealed that compared to baseline, blood glucose was significantly elevated ($p < 0.05$) after HIIE, and to the greatest extent after SIE (Figure 3.2). Furthermore, post-hoc analysis revealed that compared to baseline, blood lactate was elevated after CMIE, HIIE, and to the greatest extent after SIE (Figure 3.2).

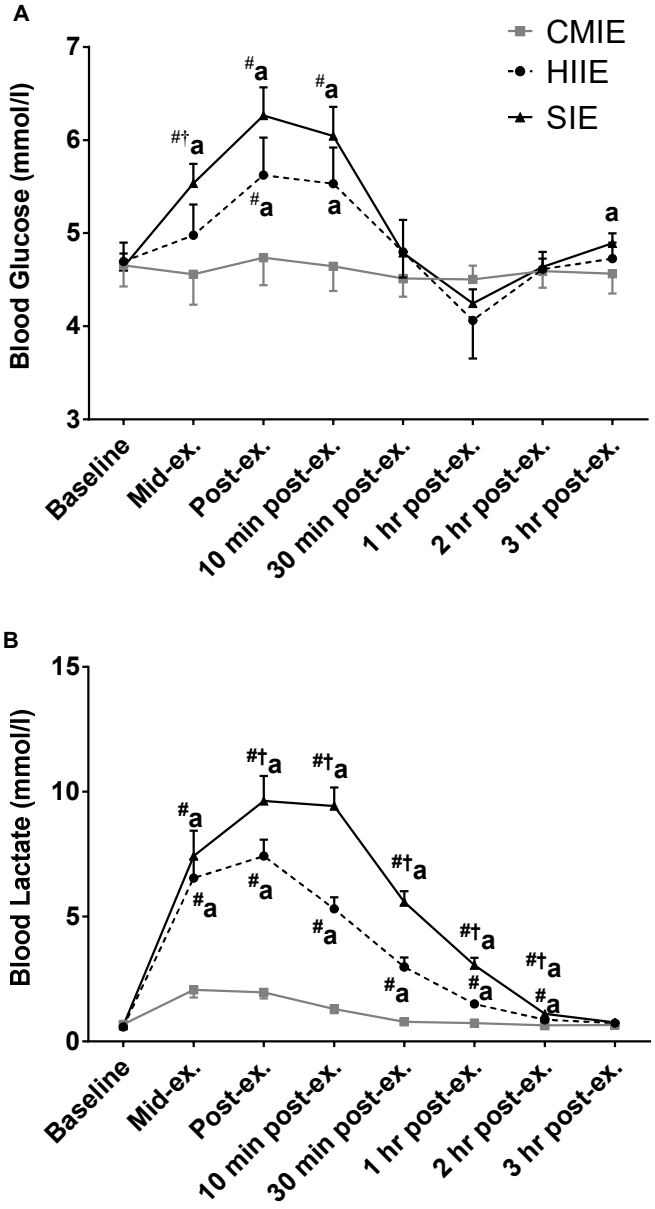

Figure 3.2: Blood lactate and blood glucose during and after exercise. Blood lactate **(A)** and blood glucose **(B)** response to high-intensity interval exercise (HIIE), sprint-interval exercise (SIE), and continuous moderate-intensity exercise (CMIE). a = $p<0.05$ compared to baseline. Significantly different ($p<0.05$) at equivalent time point vs # = CMIE and † = HIIE.

3.4.2 Plasma redox status.

A significant effect of exercise intensity ($p < 0.05$) on plasma catalase activity was detected. Post-hoc analysis indicated significantly greater plasma catalase activity with SIE compared to CMIE ($p < 0.05$), and tended to be higher compared to HIIE ($p = 0.057$; Figure 3.3). Significant time effects for hydrogen peroxide, TBARS, and SOD activity were detected (all $p < 0.05$). Post-hoc

analysis revealed greater hydrogen peroxide immediately post-exercise and 1-hour post-exercise compared to baseline; and lower TBARS at 1 and 2 hours post-exercise (Figure 3.3). SOD activity was significantly lower ($p < 0.05$) immediately, 1 hour, 2 hours, and 3 hours after exercise compared to baseline (Figure 3.3).

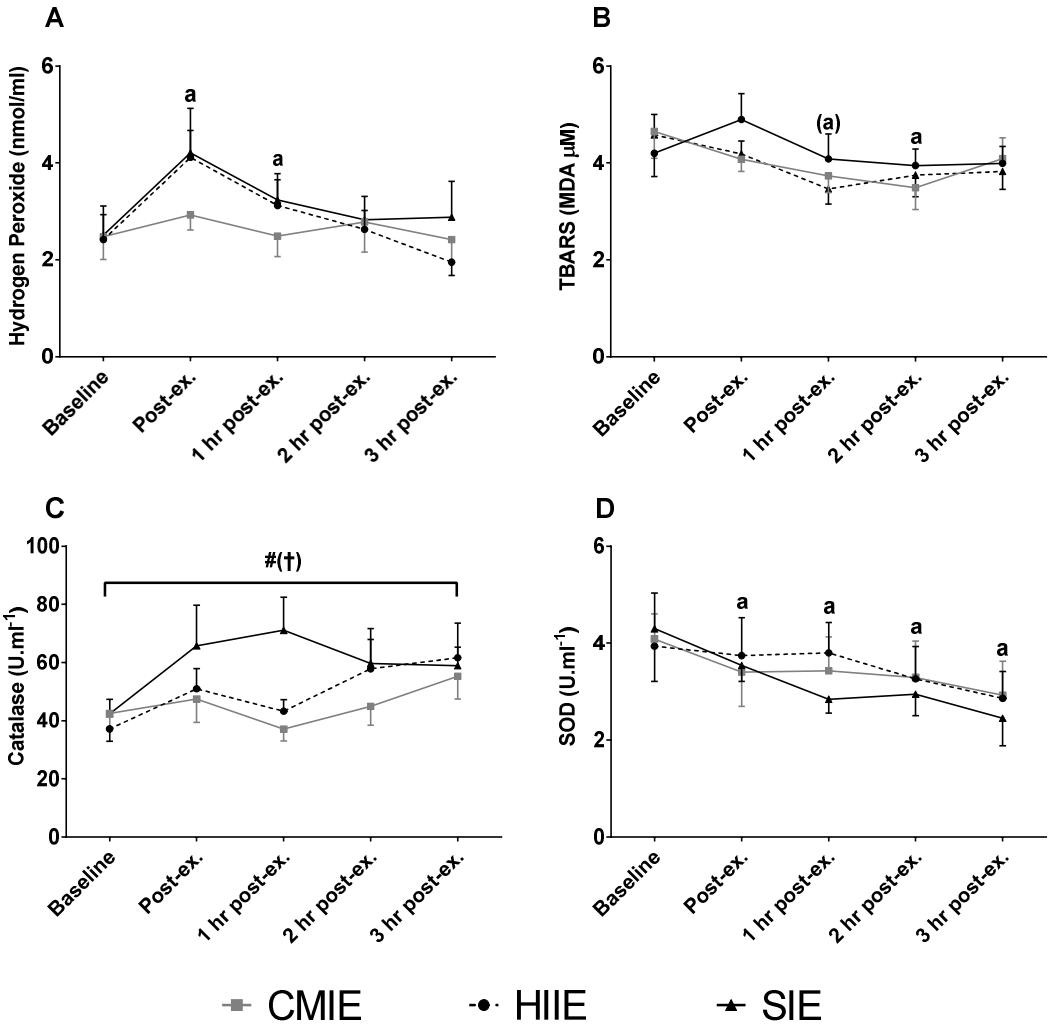

Figure 3.3: Exercise-induced plasma oxidative stress and antioxidant defence. Plasma hydrogen peroxide **(A)**, TBARS **(B)**, catalase activity **(C)**, and SOD activity **(D)**, after high-intensity interval exercise **(HIIE)**, sprint interval exercise **(SIE)**, and continuous moderate-intensity exercise **(CMIE)**. Main effect of time: a = $p < 0.05$ and (a) $p<0.1$ compared to baseline. Main exercise effect: SIE significantly different ($p < 0.05$) to $^{\#}$CMIE, and tended to be different ($p = 0.057$) to $^{(\dagger)}$HIIE. **SOD:** Superoxide dismutase. **TBARS:** thiobarbituric acid reactive substances.

3.4.3 Skeletal muscle SAPK signalling.

A significant interaction effect ($p < 0.05$) was detected for NF-κB p65 phosphorylation. Post-hoc analysis revealed significantly greater ($p < 0.05$) NF-

κB p65 phosphorylation immediately after SIE compared to baseline, and greater phosphorylation immediately after SIE compared to both HIIE and CMIE (Figure 3.4). Post-hoc analysis of main effects of time ($p < 0.05$) revealed greater phosphorylation of p38 MAPK immediately after exercise, and greater JNK phosphorylation immediately after and 3 hours after exercise compared to baseline (Figure 3.4). Post-hoc analysis of a main effect of time ($p < 0.05$) revealed lower protein abundance of IκBα immediately and 3 hours after exercise compared to baseline (Figure 3.4).

3.4.4 Skeletal muscle insulin protein signalling.

A significant interaction effect ($p < 0.05$) was detected for IRS-1^{Ser307} phosphorylation. Post-hoc analysis revealed significantly greater IRS-1^{Ser307} phosphorylation immediately after all exercise bouts, and a trend for phosphorylation to remain elevated 3 hours after CMIE (Figure 3.5). IRS-1^{Ser307} phosphorylation was significantly greater immediately after HIIE compared to CMIE, and greater 3 hours after CMIE compared to SIE. A significant interaction effect ($p < 0.001$) was detected for AS160^{Ser588} phosphorylation. Post-hoc analysis revealed lower phosphorylation of AS160^{Ser588} immediately after SIE and HIIE compared to baseline, and 3 hours after CMIE and HIIE compared to baseline (Figure 3.5). AS160^{Ser588} phosphorylation was lower immediately after SIE compared to HIIE and CMIE, and was higher 3 hours after SIE compared to CMIE. Phosphorylation of Akt^{Ser473} was lower immediately after exercise compared to baseline and tended to remain lower 3 hours after exercise (Figure 3.5). Despite increased IRS-1^{Ser307} phosphorylation, total IRS-1 protein content was not significantly influenced by exercise (Figure 3.5).

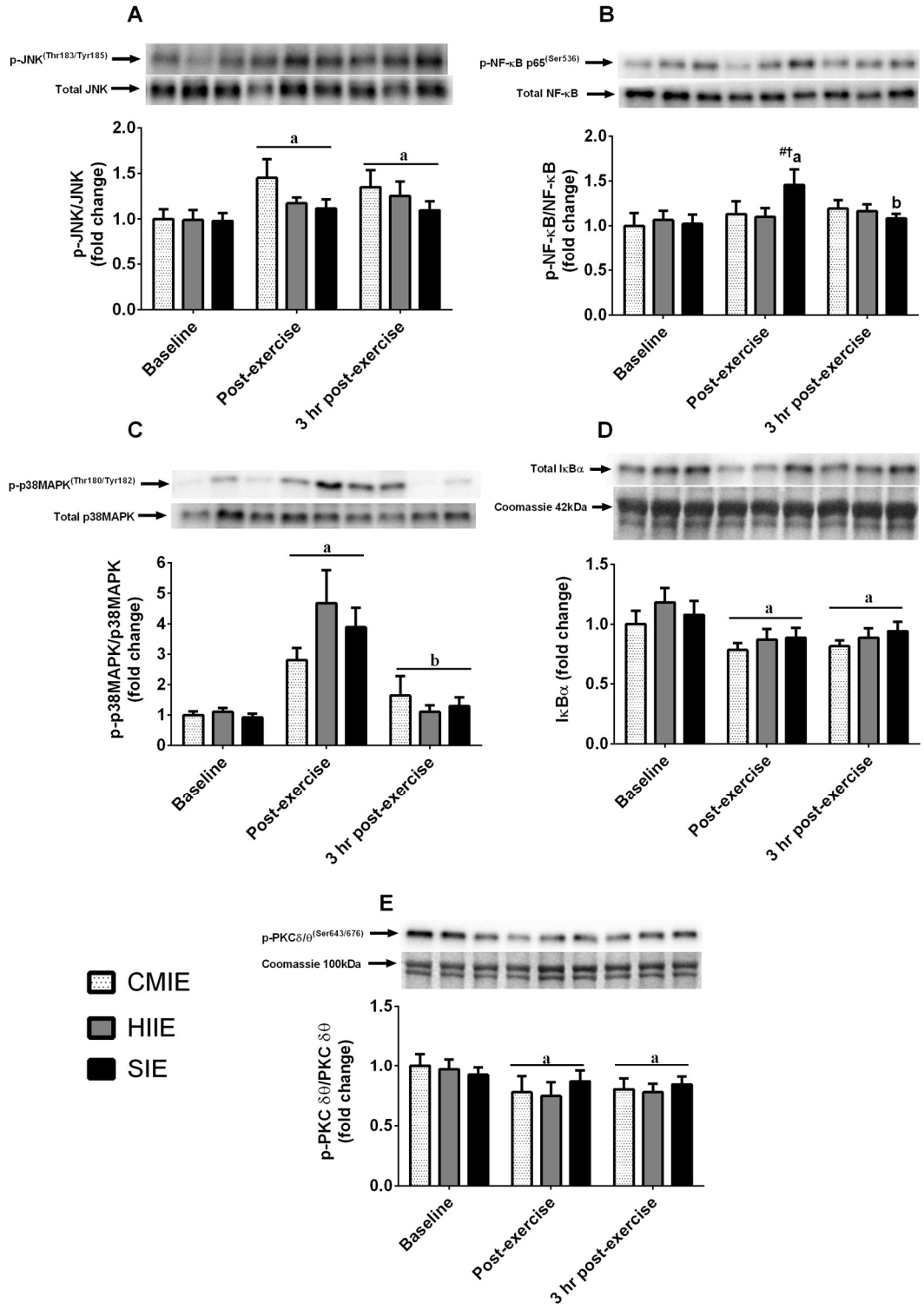

Figure 3.4: Skeletal muscle SAPK signalling. Skeletal muscle protein phosphorylation relative to total protein content of JNK$^{Thr183/Tyr185}$ **(A)**, NF-κB p65^{Ser536} **(B)**, p38 MAPK$^{Thr180/Tyr182}$ **(C)**, total protein content of IκBα **(D)**, and phosphorylated PKC δ/θ $^{Ser643/676}$ relative to coomassie protein content **(E),** after high-intensity interval exercise **(HIIE)**, sprint interval exercise **(SIE)**, and continuous moderate-intensity exercise **(CMIE)**. a = $p < 0.05$ compared to baseline; b = $p < 0.05$ compared to post-exercise. Significantly different ($p < 0.05$) at equivalent time point vs # = CMIE and † = HIIE. Representative western blot data for a single participant is provided at the top of each graph. These images correspond to the below columns and depict from left to right the three baseline, post-exercise, and 3-hour post-exercise samples for CMIE, HIIE, and SIE, respectively.

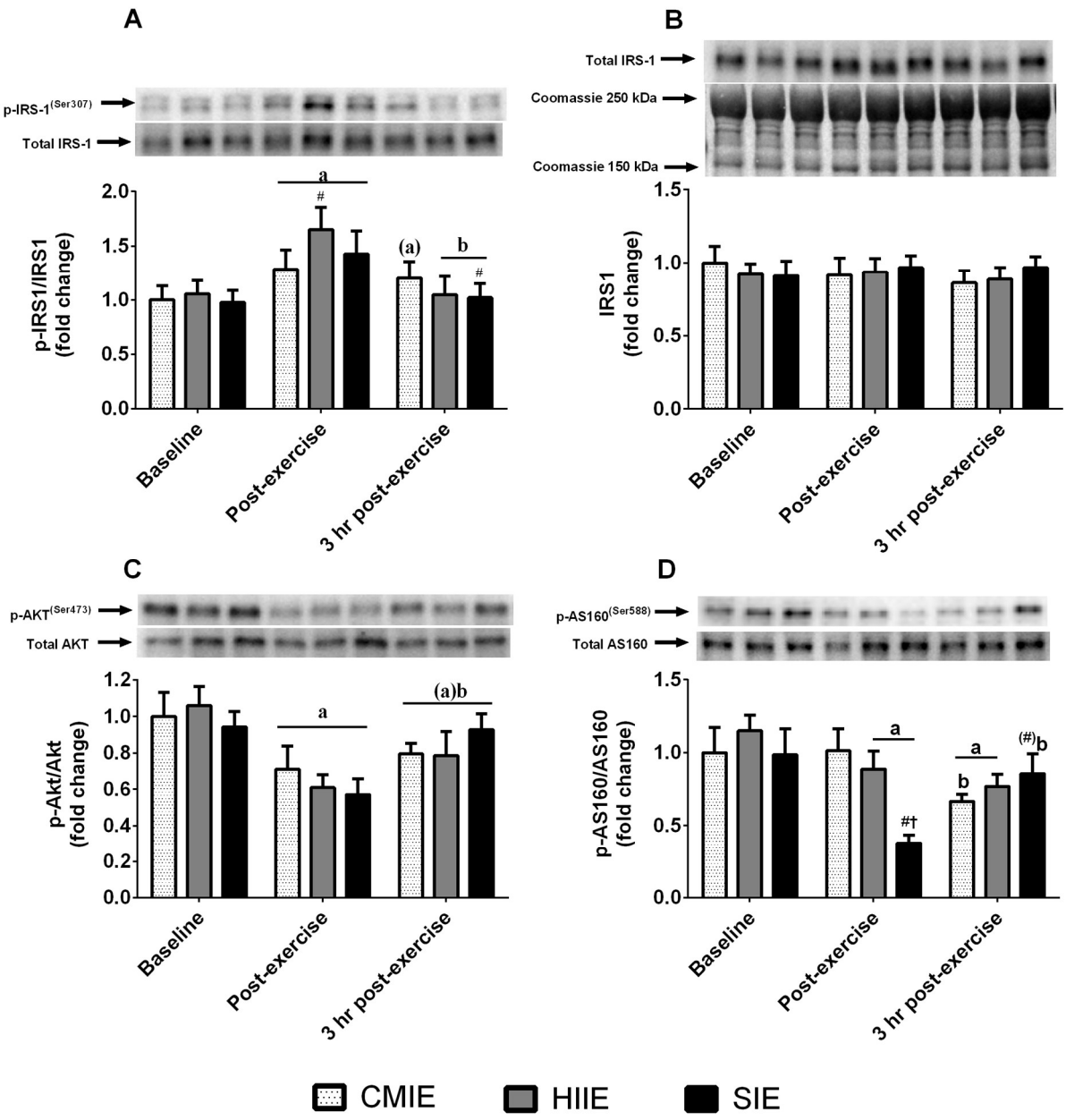

Figure 3.5: Skeletal muscle insulin protein signalling. Skeletal muscle total IRS-1 content **(B)** and phosphorylation relative to total protein content of IRS-1^{Ser307} **(A)**, Akt^{Ser473} **(C)**, and AS160^{Ser588} **(D)**, after high-intensity interval exercise **(HIIE)**, sprint interval exercise **(SIE)**, and continuous moderate-intensity exercise **(CMIE)**. a = $p < 0.05$ and (a) $p < 0.1$ compared to baseline; b = $p < 0.05$ and (b) $p < 0.1$ compared to post-exercise. Significantly different ($p < 0.05$) or trend ($p < 0.1$ in parenthesis) at equivalent time point vs # = CMIE and † = HIIE. Representative western blot data for a single participant is provided at the top of each graph. These images correspond to the below columns and depict from left to right the three baseline, post-exercise, and 3-hour post-exercise samples for CMIE, HIIE, and SIE, respectively.

3.5 Discussion.

We report that a single session of SIE elicited greater skeletal muscle NF-κB p65 phosphorylation compared to HIIE and CMIE, a similar increase in JNK and p38 MAPK phosphorylation, and a similar decrease in skeletal muscle IκBα protein content. Thus, despite consisting of less total work than CMIE and HIIE, SIE proved to be an effective stimulus for the alteration of plasma redox homeostasis and activation of redox-sensitive protein kinase signalling pathways linked to exercise-mediated adaptation of skeletal muscle.

3.5.1 Exercise-intensity and skeletal muscle SAPK signalling.

NF-κB p65 phosphorylation in human skeletal muscle was increased immediately after SIE, but not after CMIE or HIIE. It is unclear why NF-κB p65 phosphorylation was not increased after CMIE or HIIE, as NF-κB activity/phosphorylation is increased in skeletal muscle of rodents after 1 hour of swimming and treadmill exercise (181, 183). It is possible that only intense supramaximal exercise provides sufficient stimulus to increase NF-κB p65 phosphorylation in human skeletal muscle immediately after exercise. In support, Petersen, et al. (333) reported no change in human skeletal muscle NF-κB p65 phosphorylation immediately after 45-minutes of continuous cycling (71% VO_{2peak}) or after cycling to exhaustion (92% VO_{2peak}). In addition to NF-κB p65 phosphorylation, transcriptional activity of NF-κB requires ubiquitin-dependent IκBα protein degradation, a process which permits inactive cytosolic NF-κB to translocate to the nucleus (90, 204). Our findings align with others reporting decreased IκBα protein abundance in skeletal muscle after acute exercise (244, 324, 333). This decrease appears to occur independent of NF-κB p65 phosphorylation and exercise-intensity. It is possible that our biopsy sampling times may not have captured peak NF-κB phosphorylation with CMIE and HIIE, which is increased one hour after HIIE in human skeletal muscle (324), and is reported to peak 1-2 hours after exercise in human PBMC (90) and rat skeletal muscle (184).

Attenuation of the exercise-induced skeletal muscle NF-κB p65 signalling response in humans and rodents, via allopurinol, apocynin, or n-acetylcysteine treatment/ingestion, coincides with attenuation of PGC-1α, manganese

superoxide dismutase, glutathione peroxidase, citrate synthase, and mitochondrial transcription factor A gene expression (181, 209, 333). As such, greater NF-κB p65 phosphorylation after acute SIE may contribute to the equivalent or superior skeletal muscle and cardiometabolic adaptations previously reported with SIE training (141).

The mechanisms for greater NF-κB p65 phosphorylation after SIE are unclear. Catalase activity in plasma, which strongly correlates with activity in exercised skeletal muscle (443), was greater after SIE potentially reflecting the redox-sensitive nature of NF-κB p65 signalling previously reported (181, 209). Further research is required to confirm the upstream signalling responses mediating NF-κB p65 phosphorylation after SIE.

The p38 MAPK and JNK signalling pathways play an important role in exercise-mediated mitochondrial biogenesis and antioxidant defense upregulation (4, 16, 65, 167, 385). We provide evidence that JNK and p38 MAPK phosphorylation are increased to a similar extent after SIE, CMIE, and HIIE work-matched to CMIE. These findings support previous reports of similar post-exercise p38 MAPK phosphorylation after continuous exercise work-matched to high-intensity continuous cycling (105), HIIE (29), and SIE (79). Furthermore, we showed that exercise-induced skeletal muscle JNK phosphorylation in humans does not appear to occur in an exercise-intensity and/or volume manner, contradicting previous reports in rodents (160, 268). Recently, Combes, et al. (81) reported greater phosphorylation of p38 MAPK in human skeletal muscle with intermittent cycling (30 x 1-min intervals at 70% $VO2_{peak}$; 1-minute recovery periods) compared to work and intensity matched continuous cycling (30 minutes at 70% VO_{2peak}). It was proposed that increased oscillations of the cytosolic NADH/NAD+ redox state (245) elicited through intermittent exercise may play a larger role in p38 MAPK signalling compared to the manipulation of exercise volume or intensity. It is possible that the metabolic demands induced through HIIE and SIE in this and other studies were insufficient to increase p38 MAPK, and potentially JNK phosphorylation, above that of continuous exercise (29, 79). Further research is required to confirm these findings with exercise protocols that incorporate greater metabolic disturbances.

The present findings suggest that superior skeletal muscle adaptation previously reported with HIIE and SIE when compared to CMIE (141, 251, 348, 460), may occur through protein signalling pathways independent of p38 MAPK and JNK. Nevertheless, SIE consisted of considerably less total work than HIIE and CMIE, and therefore appears to be an effective exercise mode for stimulating post-exercise skeletal muscle phosphorylation of p38 MAPK, JNK, and in particular NF-κB p65.

3.5.2 Exercise-intensity and skeletal muscle insulin protein signalling.

We provide evidence that IRS-1^{Ser307} phosphorylation is increased immediately after CMIE and SIE, and to a greater extent after HIIE. Interestingly, IRS-1^{Ser307} phosphorylation was similar to baseline 3 hours after HIIE and SIE. The physiological role of IRS-1^{Ser307} phosphorylation is unclear, as it is reported to both positively and negatively regulate downstream insulin signalling and glucose uptake (324, 457). Akt^{Ser473} phosphorylation, which is downstream of IRS-1, decreased to a similar extent after all exercise protocols. Surprisingly, further probing of the distal insulin signalling cascade revealed that phosphorylation of AS160^{Ser588} was attenuated in an exercise-intensity and post-exercise time-course dependent manner.

Phosphorylation of AS160 (also known as TBC1D4) results in GTP loading and activation of Rabs, releasing GLUT4 vesicles from intracellular compartments and promoting GLUT4 vesicle plasma membrane docking and glucose uptake (190). Serine 588 specific phosphorylation of AS160 increases with human skeletal muscle contraction, insulin stimulation via the hyperinsulinaemic-euglycaemic clamp, and may play a role in the acute post-exercise enhancement of insulin sensitivity (324, 426, 427, 448). Previous research is equivocal, with studies reporting no change (64, 194) or increased phosphorylation of AS160^{Ser588} after exercise in both rodents and humans (324, 382, 426, 441). We are the first to report decreased AS160^{Ser588} phosphorylation immediately after SIE and HIIE. Using the PAS160 antibody, which primarily detects AS160^{Thr642} but also AS160^{Ser588} (139, 374), Treebak, et al. (425) also reported a decrease in AS160 phosphorylation immediately after high-intensity continuous cycling exercise (20 minutes, 80% VO_{2peak}), whereas phosphorylation was unchanged immediately after CMIE (30 mins,

~67% VO_{2peak}). We extend previous findings by reporting that $AS160^{Ser588}$ phosphorylation is similar to baseline 3 hours after SIE, but remains lower after HIIE and CMIE.

The mechanism for the substantial decrease in $AS160^{Ser588}$ phosphorylation immediately after SIE is unclear. The reported elevation in blood glucose during and immediately after SIE, and to a lesser extent after HIIE, suggests a transient counter-regulatory hormonal response previously reported after higher-intensity exercise (266). Certainly, resistance exercise and extreme muscle damaging exercise inhibit insulin protein signalling (14, 188), likely through mTOR inhibition of the PI3K signalling pathway (176). However, mTOR signalling does not appear to be activated following acute SIE (142). Alternatively, excess ROS such as hydrogen peroxide may override the potentiation of insulin signalling through the inactivation of protein tyrosine phosphatases (74, 257, 386), by increasing JNK and NF-κB mediated inhibition of the PI3K/Akt signalling pathway (195, 287). In TNF-α/NF-κB induced insulin resistant human myotubes, targeted interference of the NF-κB signalling pathway restores insulin stimulated AS160 and Akt phosphorylation and glucose uptake, despite minimal effect on JNK phosphorylation (22). Taken together, it is possible that SIE induced NF-κB signalling may transiently suppress AS160 phosphorylation immediately after exercise. Whether the differential effect of exercise-intensity on post-exercise $AS160^{Ser588}$ phosphorylation occurs at other AS160 phosphorylation sites, and whether these changes effect post-exercise insulin sensitivity, are unknown and further explored in Chapter 4.

3.5.3 Exercise-intensity and plasma redox status.

Exercise intensity and plasma redox status. We report that SIE elicited greater post-exercise plasma catalase activity compared to HIIE and CMIE, despite a similar increase in plasma hydrogen peroxide and concomitant decrease in SOD activity and TBARS. The discrepancy between biomarkers is unclear, but may stem from the inability of some plasma redox biomarkers to accurately reflect changes in exercised skeletal muscle (443).

Wadley, et al. (450) recently reported that low-volume HIIE (10 x 1 minutes at 90% VO_{2max}) increases plasma lipid peroxidation and decreases protein

carbonyls in untrained males to a similar extent as work-matched high-intensity (20 minutes @ 80% VO_{2max}) and moderate-intensity continuous cycling protocols (27 minutes @ 60% VO_{2max}). However, redox status was only measured up to 30 minutes post-exercise (450), a time-period which may not capture peak plasma oxidative stress and antioxidant activity (280). We extend these findings demonstrating that in healthy recreationally active individuals, SIE elicits greater post-exercise catalase activity. Furthermore, SIE, HIIE, and CMIE, similarly increased plasma hydrogen peroxide for up to 1 hour after exercise, and decreased plasma TBARS and SOD activity for up to 2 and 3 hours post-exercise, respectively.

The upregulation of systemic endogenous antioxidant defense through regular exercise is reported to play an important role in attenuating chronic oxidative stress and improving cardiometabolic health (119, 156, 358). A single session of SIE provides greater protection against postprandial lipaemia and oxidative stress 24 hours later when compared to lower-intensity continuous exercise (136). Furthermore, three weeks of SIE training in heathy humans upregulates antioxidant defense, including catalase activity, and attenuates systemic oxidative stress (52). Taken together, greater plasma catalase activity after SIE may highlight a potential pathway for the reported superior cardiometabolic health benefits of SIE (141).

3.5.4 Limitations.

A potential limitation of the study is a small sample size. However, previous invasive human studies have used similar sample sizes to detect significant changes in SAPK signalling (81, 333, 420). The combined analysis of both males and females may limit interpretation of the results. Nevertheless, exercise-induced oxidative stress and p38 MAPK protein signalling appear to be similar between sexes (131). Furthermore, in the current study we did not undertake subcellular fractionation, immunohistochemistry, and/or direct measurements of kinase activity due to limited tissue availability. Protein kinase signalling is reported to be spatial-temporally sensitive (34, 204) and as such future studies are required to determine the subcellular localization of protein kinase phosphorylation and kinase activity before and after exercise of different intensities and mode. It is also important to note that the acute activation of

protein signalling pathways in skeletal muscle do not always reflect functional changes in protein synthesis and/or adaptations with chronic exercise training (79, 451). Finally, findings in this study are limited to young recreationally active adults, the specific exercise-protocols investigated, and the investigation of a single session of exercise. Future research is required to confirm these findings with subsequent bouts of exercise over a longer period of time, in more diverse populations with different exercise protocols.

3.5.5 Conclusion.

These findings demonstrate that p38 MAPK and JNK phosphorylation increase to a similar extent after CMIE, HIIE and SIE. On the other hand, skeletal muscle NF-κB phosphorylation and plasma catalase activity is more responsive to intense exercise. Whether greater NF-κB phosphorylation post-SIE contributes to the previously reported superior benefits of SIE on skeletal muscle adaption warrants further investigation. Surprisingly, only CMIE and HIIE elicited a decrease in phosphorylation of the downstream glucose uptake signalling protein AS160 three hours after exercise, despite substantially lower AS160 phosphorylation immediately after SIE. These findings indicate that the time course of post-exercise AS160 phosphorylation, an important regulator of contraction and insulin-stimulated glucose uptake, is influenced in an exercise-intensity dependent manner. Taken together, exercise-intensity plays a role in regulating the complex SAPK signalling pathways which are known to be involved in the adaptive cardiometabolic responses to exercise.

CHAPTER 4. ACUTE HIGH-INTENSITY INTERVAL EXERCISE-INDUCED REDOX SIGNALLING IS ASSOCIATED WITH ENHANCED INSULIN SENSITIVITY IN OBESE MIDDLE-AGED MEN.

Data presented within this chapter has been published in manuscript form and has been adapted for presentation in this thesis:

Parker L, Stepto N, Shaw C, Serpiello F, Anderson M, Hare D, and Levinger I. Acute high-intensity interval exercise-induced redox signalling is associated with enhanced insulin sensitivity in obese middle-aged men. *Frontiers in Physiology* 7: 411, 2016.

Levinger I, Jerums G, Stepto NK, **Parker L,** Serpiello FR, McConell GK, Anderson M, Hare DL, Byrnes E, Ebeling PR, and Seeman E. The effect of acute exercise on undercarboxylated osteocalcin and insulin sensitivity in obese men. *J Bone Miner Res* 29: 2571-2576, 2014.

This study was in-part funded by a Gatorade Sports Science Institute student grant.

4.0 General introduction.

Chapter 3 explored the effects of HIIE, SIE, and CMIE on post-exercise plasma redox status and skeletal muscle SAPK and glucose uptake protein signalling. Findings established that SIE elicited greater post-exercise plasma catalase activity and NF κB-p65 signalling in skeletal muscle, whereas phosphorylation of p38 MAPK, JNK and PKC δ/θ, were similar between exercise protocols. Surprisingly, SIE induced greater impairment of the downstream glucose uptake protein AS160^{Ser588} immediately after exercise, whereas phosphorylation was lower with CMIE and HIIE 3 hours after exercise. Additionally, only CMIE induced IRS-1^{Ser307} phosphorylation 3 hours after exercise despite greater IRS-1^{Ser307} phosphorylation immediately after exercise with HIIE. Thus, it appears that post-exercise SAPK and glucose uptake protein signalling occurs in an exercise intensity and post-exercise time-course dependent manner. These findings develop our understanding of the effects of exercise-intensity on SAPK and glucose uptake protein signalling in humans. However, the impact on insulin sensitivity was not assessed, limiting the translation of findings to health and disease. Furthermore, muscle biopsy sampling times may not have been suitable to capture peak post-exercise phosphorylation of SAPK and/or glucose uptake protein signalling. Previous research has also reported greater basal oxidative stress, exercise-induced oxidative stress, and basal activation of SAPK signalling pathways in populations at risk of cardiometabolic disease. Therefore, the aims of Chapter 4 were to measure the effect of a single session of HIIE and insulin stimulation, on post-exercise redox status, SAPK and insulin protein signalling, and insulin sensitivity assessed by hyperinsulinaemic-euglycaemic clamp in obese middle-aged males.

4.1 Abstract.

Background. Obesity and ageing are associated with increased oxidative stress, activation of stress and mitogen activated protein kinases (SAPK), and the development of insulin resistance and metabolic disease. In contrast, acute exercise also increases oxidative stress and SAPK signalling, yet is reported to enhance insulin sensitivity and reduce the risk of metabolic disease. This study explored this paradox by investigating the effect of a single session of high-intensity interval-exercise (HIIE) on redox status, muscle SAPK and insulin protein signalling in eleven middle-aged obese men. **Methods.** Participants completed a 2 hour hyperinsulinaemic-euglycaemic clamp at rest, and 60 minutes after HIIE (4x4 mins at 95% HR_{peak}; 2 min recovery periods), separated by 1-3 weeks. **Results.** Irrespective of exercise-induced changes to redox status, insulin stimulation both at rest and after HIIE similarly increased plasma superoxide dismutase activity, plasma catalase activity, and skeletal muscle 4-HNE; and significantly decreased plasma TBARS and hydrogen peroxide. The SAPK signalling pathways of p38 MAPK, NF-κB p65, and JNK, and the distal insulin signalling protein $AS160^{Ser588}$, were activated with insulin stimulation at rest and to a greater extent with insulin stimulation after a prior bout of HIIE. Higher insulin sensitivity after HIIE was associated with higher insulin-stimulated SOD activity, JNK, p38 MAPK and NF-kB phosphorylation ($r=0.63$, $r=0.71$, $r=0.72$, $r=0.71$; $p<0.05$, respectively). **Conclusion.** These findings support a role for redox homeostasis and SAPK signalling in insulin-stimulated glucose uptake which may contribute to the enhancement of insulin sensitivity in obese men 3 hours after HIIE.

4.2 Introduction.

Obesity is a major risk factor for insulin resistance and type 2 diabetes. Regular physical activity improves glycaemic control and as such is a key lifestyle goal for the prevention and management of obesity and type 2 diabetes (258). Even a single session of exercise can enhance insulin sensitivity in the hours after exercise (127). However, the mechanisms by which exercise improves insulin sensitivity are not completely understood, but may include oxidation-reduction

(redox) reactions and their inherent capacity to both impair and/or facilitate insulin signalling and insulin-stimulated glucose uptake (421).

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and endogenous antioxidant defences (346). While modest increases in ROS are regulated by endogenous antioxidants, certain ROS-inducing stimuli, such as exercise, diet, age and disease, may overpower these systems in favour of oxidative stress (436). Chronic systemic oxidative stress is associated with obesity, insulin resistance and type 2 diabetes (30, 436). Oxidative stress-induced insulin resistance can occur through protein modification via both lipid peroxidation and the activation of stress and mitogen activated protein kinase signalling (SAPK) pathways (416, 421). Sustained activation of these redox-sensitive signalling pathways leads to inhibitory phosphorylation of the insulin receptor substrate 1 (IRS-1) at human serine residues 312 and 307, promoting IRS-1 degradation, impaired insulin signalling and attenuation of insulin-stimulated glucose uptake (15, 95, 166, 336, 416, 459). The prevention of IRS-1 degradation through the inhibition of ROS and/or SAPK signalling has been shown to restore insulin signalling and insulin-stimulated glucose uptake (138, 336, 398, 421). Collectively, these studies suggest a pathological role of redox induced lipid peroxidation and SAPK signalling in aberrant insulin signalling and subsequent insulin resistance.

Paradoxically, ROS produced during and after muscular contraction also transiently activate SAPK signalling pathways and lipid peroxidation (228), however during this period glucose uptake and insulin sensitivity are reported to be enhanced (127). An accumulation of research now suggests that depending on the biological context, redox signalling is integral for optimal physiological functioning and adaptation to physiological stress (346). Redox-signalling may play an important role in contraction-induced (373) and insulin-stimulated glucose uptake (30, 219, 253, 429); whether these redox-signalling pathways are activated with insulin stimulation after a single session of exercise are unknown. High-intensity interval exercise (HIIE) is an effective exercise mode for improving glycaemic control in clinical populations (141, 251), however the impact of acute HIIE on redox-sensitive protein signalling and insulin sensitivity in obese middle-aged males is unknown. The aim of this study

was to test the hypothesis that a single session of HIIE would transiently increase oxidative stress and SAPK signalling and insulin signalling which may, at least in part, be related to post-exercise enhancement of insulin sensitivity in middle-aged, obese males.

4.3 Methodology and procedures.

4.3.1 Participants.

Eleven middle-aged (58.1±2.2 years mean±SEM, range 40-70 years), obese men (BMI = 33.1±1.4 $kg \cdot m^{-2}$), without diabetes (fasting glucose: 5.3±0.2 $mmol \cdot L^{-1}$ and $HbA1_C$: 5.6±0.1%; 34±1.1 mmol/mol), participated in the study (242). Verbal and written explanations about the study were provided to participants prior to providing written consent. Exclusion criteria for participation included medications known to affect insulin secretion and/or insulin sensitivity; musculoskeletal or other conditions that prevent daily activity; and symptomatic or uncontrolled metabolic or cardiovascular disease. This study was approved by and carried out in accordance with the Victoria University Human Research Ethics Committee for experiments involving humans.

4.3.2 Screening and preliminary testing.

Participants were asked to complete a symptom limited incremental cycle VO_{2peak} determination test as previously described (241). Ventilatory expired gas (15 second averages) was collected from each participant and analysed using a metabolic cart (Medgraphics, Cardio2 and CPX/D System, USA).

4.3.3 Study design.

Participants abstained from food (overnight fast), physical activity (72 hours), and alcohol and caffeine consumption (24 hours) prior to each trial day. To avoid glycogen depletion volunteers were provided dietary information and asked to consume approximately 300g of carbohydrate 24 hours prior to their first trial. This was recorded in a diet diary and replicated for their second trial. For the main experimental trial participants completed a 2 hour hyperinsulinaemic-euglycaemic clamp (insulin clamp) at rest (rest trial), and 60 minutes after HIIE (exercise trial), separated by 1-3 weeks (Figure 4.1).

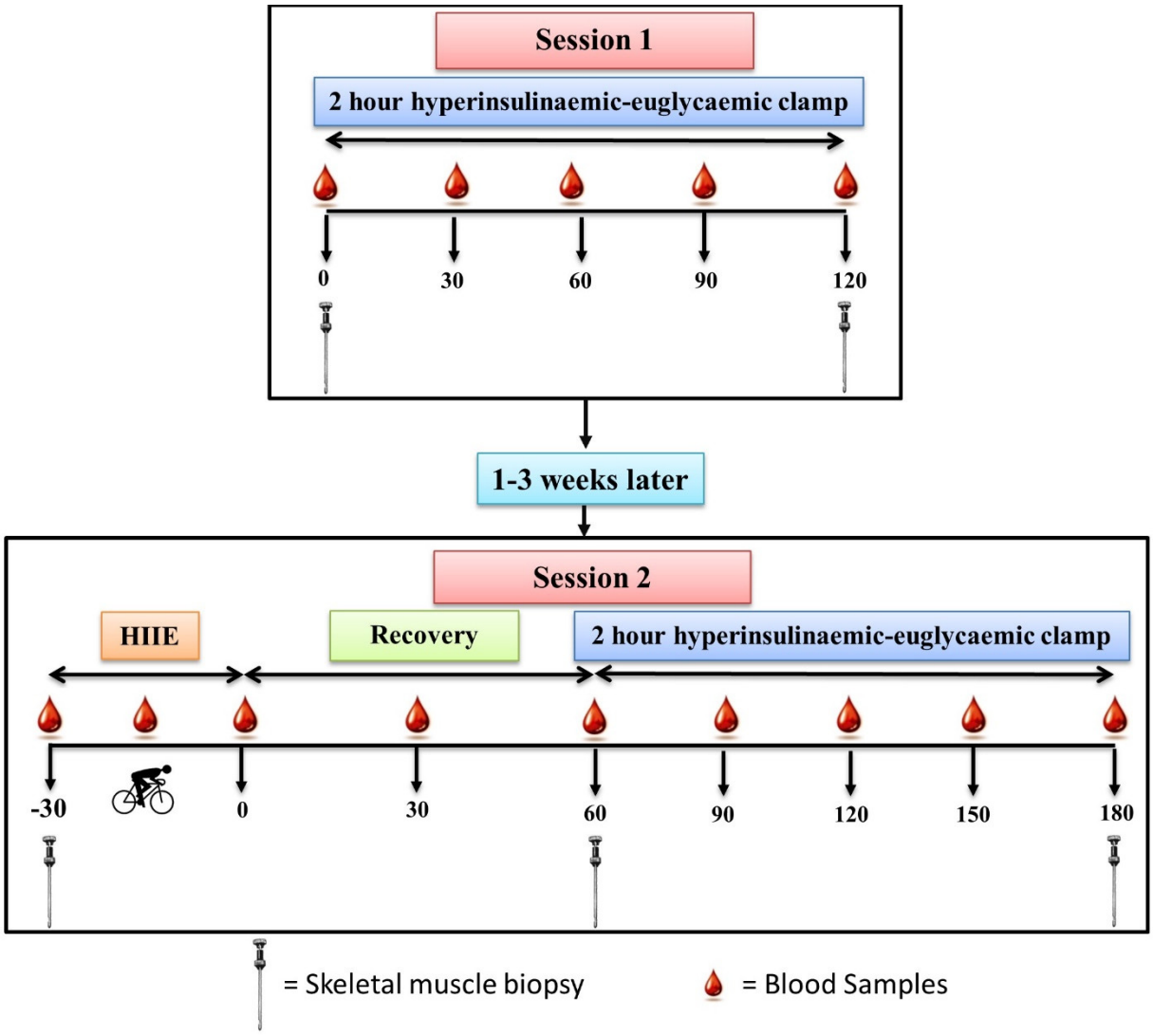

Figure 4.1: Schematic outline of research methodology for study 2. After initial screening, participants arrived in the morning after an overnight fast and underwent a 2 hour hyperinsulinaemic-euglycaemic clamp. A minimum of 1 week later, participants arrived in the morning after an overnight fast and underwent 30 minutes of combined high-intensity interval exercise (HIIE). After one hour of recovery, a 2 hour hyperinsulinaemic-euglycaemic clamp was performed as per the first session. Skeletal muscle and blood samples were taken through the study as indicated in the diagram.

4.3.4 Rest trial.

Participants arrived in the morning following an overnight fast and a 2-hour hyperinsulinaemic-euglycaemic clamp was performed to measure baseline insulin sensitivity via methods previously reported (191, 242, 407). In brief, insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused at 40mU.m^{-2} per minute for 120 minutes inducing a stable state of hyperinsulinemia (242). Concomitantly, exogenous glucose was infused at a rate necessary to maintain a stable blood glucose reading of ~5 mmol.l^{-1} which

was assessed every 5 minutes during the insulin clamp with an automated analyser (YSI 2300 STAT Plus Glucose & Lactate Analyser, USA). Insulin sensitivity was calculated by averaging the glucose infusion rate (GIR, $mg \cdot kg^{-1} \cdot min^{-1}$) over the final 30 minutes of the insulin clamp and normalized to serum insulin levels (M-Value) (187). Muscle biopsies and venous blood samples were taken at baseline and after the 2-hour insulin clamp.

4.3.5 Exercise trial.

Approximately one to three weeks later participants arrived after an overnight fast and performed 30 minutes of HIIE on a Lode cycle ergometer (Corvial, Lode B.V., Groningen, The Netherlands). The HIIE included a 4-minute warm-up at 50% to 60% HR_{peak}, followed by 4 x 4-minute cycling bouts at 95% HR_{peak}, interspersed with 2 minutes active recovery at 50% to 60% HR_{peak}. The target HR for the exercise session was determined by the heart rate reserve method using the following formula: exercise target HR = % of target intensity (HR_{peak} – HR_{rest}) + HR_{rest}. After the exercise bout participants underwent 1 hour of passive recovery after which the 2-hour insulin clamp was performed as per the previous trial.

4.3.6 Skeletal muscle and blood sampling.

Vastus lateralis muscle and venous blood samples were taken at baseline and after the insulin clamp in the rest trial; and at baseline, pre-insulin clamp (1-hour post-exercise) and post-insulin clamp (three hours post-exercise) in the exercise trial. Muscle samples were obtained from the vastus lateralis under local anaesthesia (Xylocaine 1%) utilizing a Bergström needle with suction (113). The samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Venous blood was collected from an antecubital vein (contra-lateral to infusions) via an intravenous cannula and appropriate collection tubes. Blood samples were centrifuged at 3,500 rpm for 15 minutes at 4°C and plasma subsequently aliquoted and stored at -80°C until analysed.

4.3.7 Plasma redox status analysis.

Plasma thiobarbituric acid reactive substances (TBARS), catalase activity, superoxide dismutase (SOD) activity, and Hydrogen Peroxide (Molecular Probes, USA) were analysed as per methods outlined in Chapter 3. Intra-assay

coefficients of variation were determined and averaged from each duplicate for all participants and resulted in a coefficient of variation of 1%, 2%, 5% and 2% for TBARS, SOD, catalase and hydrogen peroxide, respectively. Inter-assay coefficients of variation for assay standards between each 96 well plate were averaged and resulted in a coefficient of variation of 1%, 4%, 2%, 1%, for TBARS, SOD, catalase and hydrogen peroxide, respectively.

4.3.8 Skeletal muscle protein analysis.

Skeletal muscle protein analysis was performed as per the methods outlined in Chapter 3. In brief, cryosections of skeletal muscle were homogenized and protein content determined (296). Lysate was loaded into a 7.5% pre-cast gel and proteins separated by electrophoresis and transferred to polyvinylidene difluoride membranes. After appropriate blocking and washing, membranes were incubated overnight with the following primary antibodies: phospho-SAPK/JNK (Thr183/Tyr185; Cell Signalling Technology; CST #9251), SAPK/JNK (CST #9252), phospho-p38 MAPK (Thr180/Tyr182; CST #9211), p38 MAPK (CST #9212), phospho-NF-κB p65 (Ser536; CST #3033), NF-κB p65 (CST #8242), IκBα (CST #4814), phospho-PKC δ/θ (Ser643/676; CST #9376), phospho-IRS-1(Ser307; CST #2384), phospho-AS160 (Ser318; CST #8619), phospho-AS160 (Ser588; CST #8730), phospho-Akt (Ser473; CST #9271), Akt (CST #9272), phospho-AS160 (Thr642; CST #4288), phospho-GSK-3α/β (Ser21/9; CST #9331), AS160 (CST #2447), 4-Hydroxynonenal (Abcam, ab46545) and IRS-1 (Millipore, 06-248). After incubation, membranes were washed, incubated at room temperature with horseradish peroxidase conjugated secondary antibody, re-washed and incubated in SuperSignal West Femto Maximum Sensitivity substrate for 5 minutes. Protein densitometry was measured and normalized to total protein content values obtained from the Coomassie staining protocol. Where appropriate, phosphorylated proteins are expressed relative to total specific protein content.

4.3.9 Statistical analysis and sample size calculation.

Data were checked for normality and analysed using Predictive Analytics Software (PASW, USA). Comparisons of means at baseline and after insulin stimulation in the rest and exercise trial were examined using a two way (intervention x time point) repeated measures analysis of variance (ANOVA).

Comparisons of multiple means within the exercise trial were examined using a one way repeated measures ANOVA. Post hoc analysis of significant interaction and main effects were performed using Fisher's protected LSD test. Associations between insulin sensitivity, redox status and SAPK signalling were analysed using Pearson's coefficient of correlation. Cook's Distance was used as a measure of influence where observations greater than one Cook's D were excluded from correlation analysis (83). All data are reported as mean ± standard error of mean (SEM) and all statistical analysis were conducted at the 95% level of significance ($p \leq 0.05$). Trends were reported when p-values were greater than 0.05 and less than 0.1.

A priori calculations (G*Power 3.1. 9) indicated that with an alpha set at 0.05 and power at 0.8, a sample size of eight was required to detect a significant ($p < 0.05$) improvement in the insulin sensitivity index ~5 hours after acute cycling aerobic exercise. This calculation was based on a previously reported effect size f of 0.75 (mean improvement of 1.2 ± 2.12 $mg \cdot kg^{-1} \cdot min^{-1} \cdot$insulin mU/l^{-1}; n = 7) (187). Thus, the study was adequately powered for the main outcome measure of post-exercise enhancement of insulin sensitivity.

4.4 Results.

4.4.1 Insulin sensitivity.

Exercise significantly increased insulin sensitivity (glucose infusion rate and m-value) by ~34-40% compared to the rest trial (Figure 4.2).

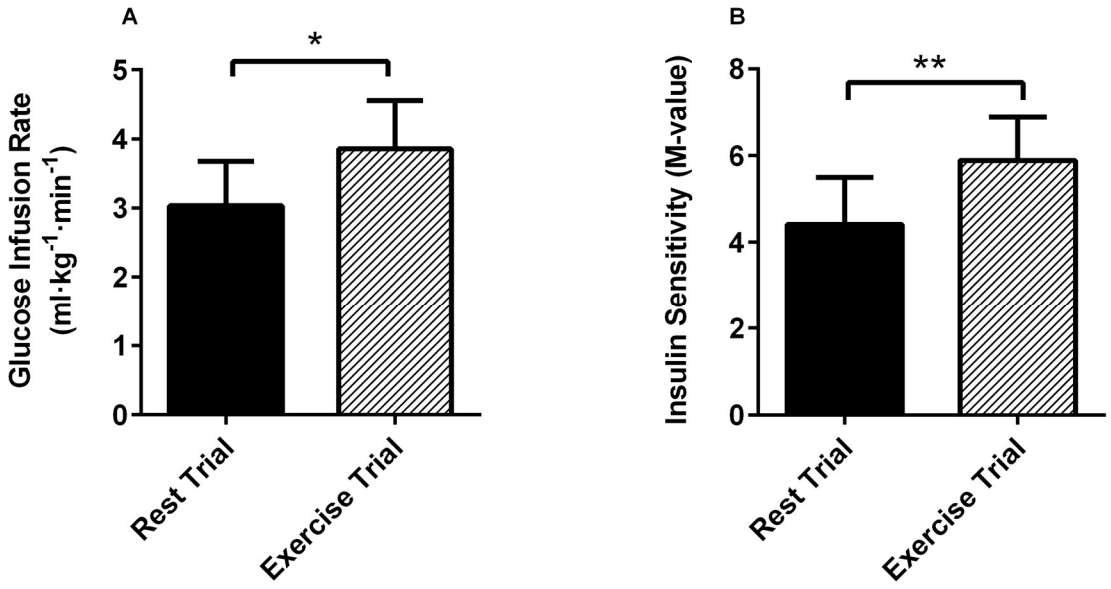

Figure 4.2: Post-exercise insulin sensitivity. Glucose infusion rate **(A)** and insulin sensitivity (M-Value; **B**) measured via a 2 hour hyperinsulinaemic-euglycaemic clamp at rest, and approximately 3 hours after a single session of high-intensity interval exercise. *p < 0.05 and **p < 0.01 are significantly different. Adapted from Levinger, et al. (242).

4.4.2 Plasma redox status.

One hour after the HIIE session (prior to insulin stimulation) catalase activity significantly increased and TBARS and hydrogen peroxide decreased (Figure 4.3). Insulin stimulation both at rest and after exercise elicited a similar decrease in plasma hydrogen peroxide and TBARS, and increase in plasma catalase and superoxide dismutase activity (Figure 4.3).

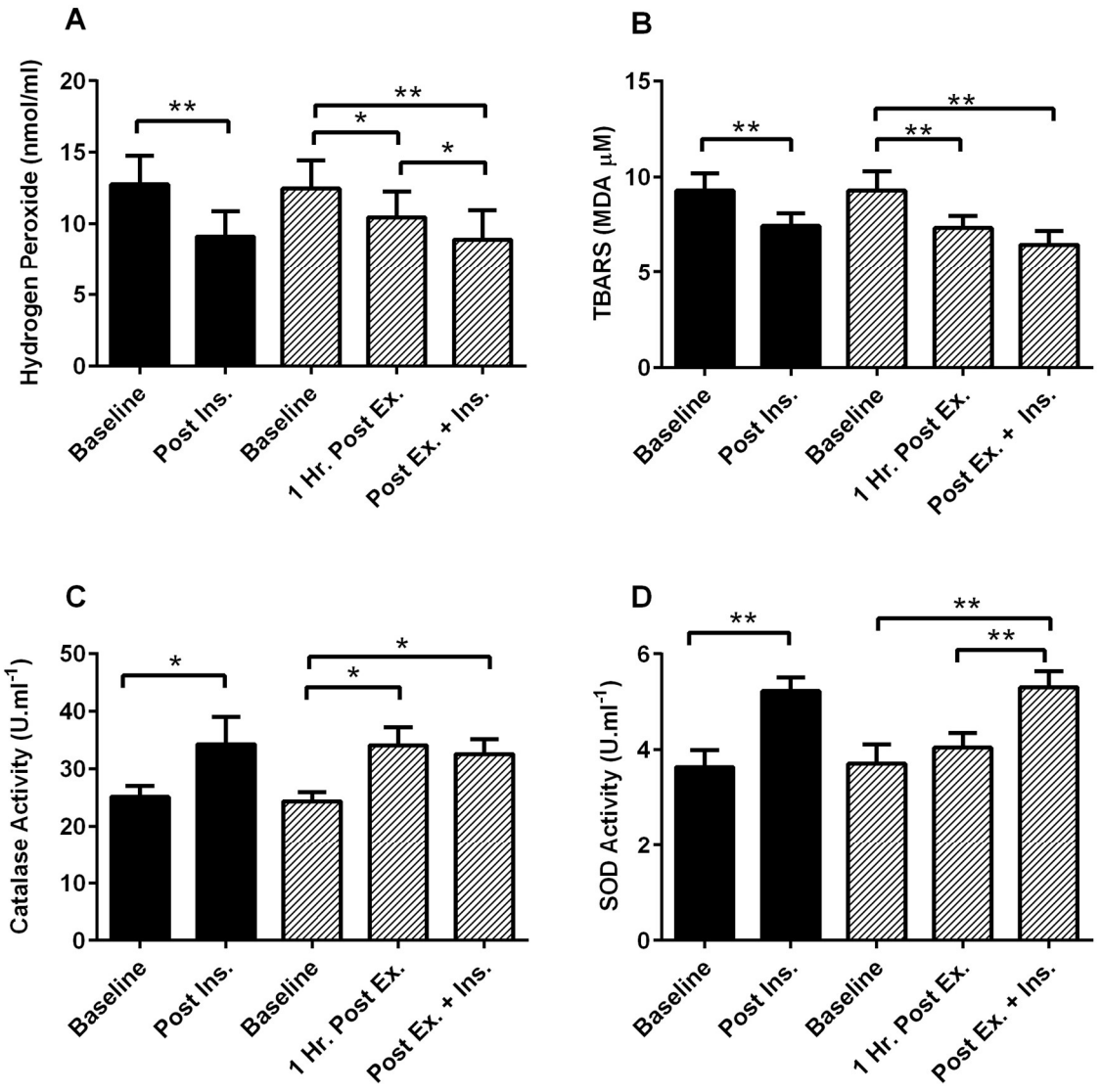

Figure 4.3: Exercise, insulin stimulation, and plasma redox status. Oxidative stress and antioxidant enzymatic activity biomarkers in plasma 1 hour after exercise (prior to insulin stimulation), and pre and post insulin stimulation at rest and 3 hours after exercise (n = 11). **A**: Hydrogen Peroxide; **B**: Thiobarbituric acid reactive substances (TBARS); **C**: Catalase activity; **D**: Superoxide dismutase activity (SOD). *p < 0.05 and **p < 0.01 are significantly different. Black bars = rest trial. Diagonal line bars = exercise trial. Ex. = High-intensity interval exercise. Ins. = insulin stimulation via the hyperinsulinaemic-euglycaemic clamp.

4.4.3 Skeletal muscle SAPK signalling.

The acute session of exercise (prior to insulin stimulation) significantly increased phosphorylation of JNK$^{Thr183/Tyr185}$, p38 MAPK$^{Thr180/Tyr182}$, NF-κB p65^{Ser536}, GSK-3α/β$^{Ser21/9}$, and 4-HNE protein modification (Figures 4.4 and 4.5). In contrast, phosphorylated PKC δ/θ$^{Ser643/676}$ was significantly lower after exercise (Figure 4.5). Insulin stimulation in the rest trial significantly increased

phosphorylation of JNK$^{Thr183/Tyr185}$, p38 MAPK$^{Thr180/Tyr182}$, and 4-HNE protein modification (Figure 4.4). The prior bout of HIIE significantly increased insulin-stimulated phosphorylation of JNK, p38 MAPK and NF-κB p65 to a greater extent. Total protein content of IκBα was significantly lower after insulin stimulation in both the rest and HIIE trial (Figure 4.4). PKC δ/θ phosphorylation remained lower compared to baseline. There was a tendency for increased phosphorylation of GSK-3α/β after insulin stimulation in both the rest and exercise trial (Figure 4.5).

4.4.4 Skeletal muscle insulin signalling.

Insulin stimulation both at rest and after a HIIE elicited a similar increase in phosphorylation of IRS-1^{Ser307}, AS160^{Ser318} and AS160^{Thr642} (Figure 4.6). Phosphorylation of Akt^{Ser473} and AS160^{Ser588} were increased 1 hour after exercise, and were phosphorylated to the greatest extent with insulin stimulation after HIIE.

4.4.5 Insulin sensitivity associations.

Insulin sensitivity (m-value) following exercise was positively associated with higher levels of insulin-stimulated SOD activity ($r=0.634$, $p=0.036$, $n=11$), phosphorylated-JNK ($r=0.709$, $p=0.007$, $n=10$), p38 MAPK ($r=0.724$, $p=0.018$, $n=10$) and NF-κB p65 ($r=0.708$, $p=0.022$, $n=10$), and tended to correlate with lower levels of phosphorylated PKC δ/θ ($r=-0.571$, $p=0.066$, $n=11$). No correlations between insulin sensitivity and the variables of interest were detected in the rest trial ($p>0.05$).

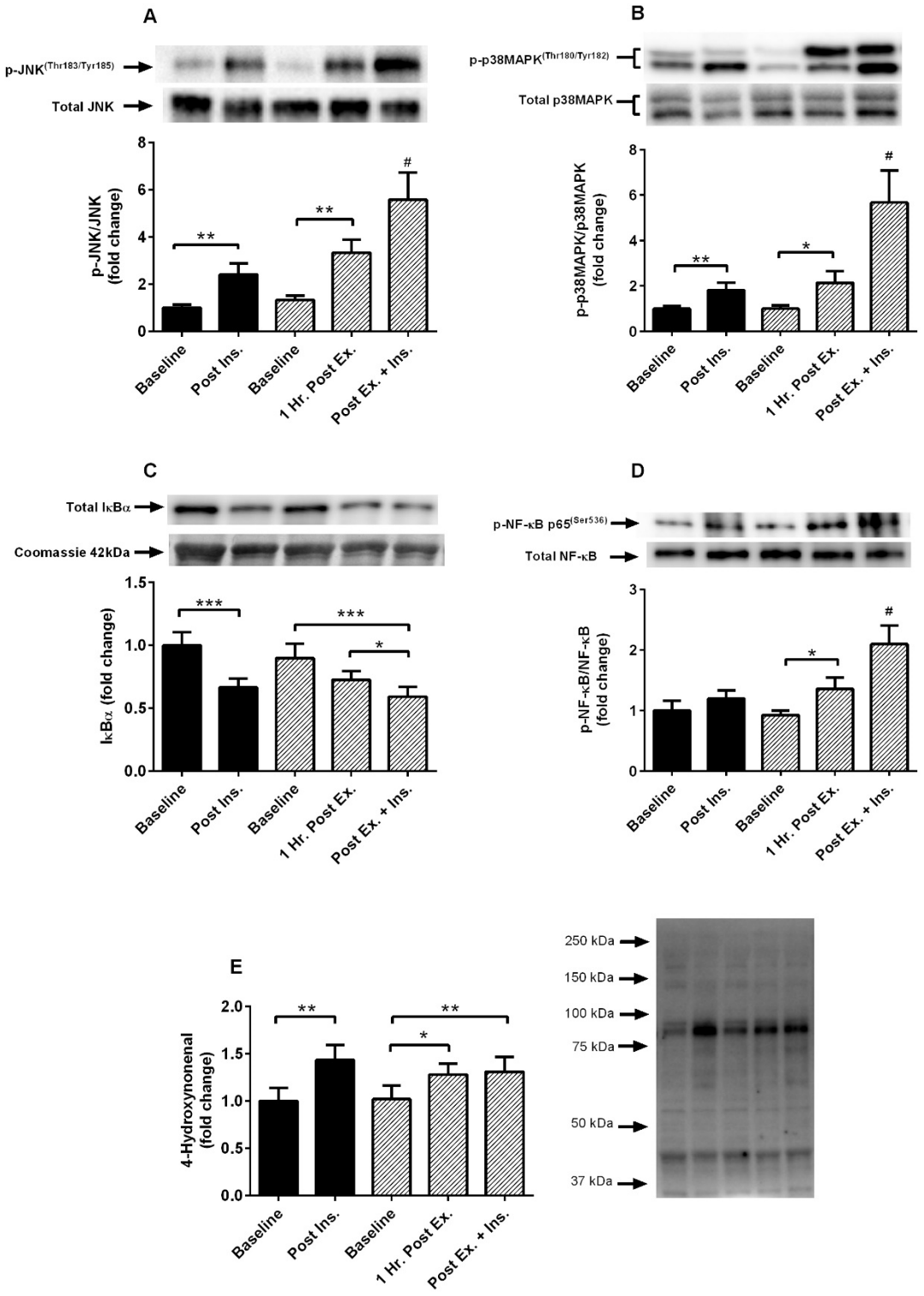

Figure 4.4: Exercise, insulin stimulation, and skeletal muscle SAPK signalling. Phosphorylation relative to total protein content in skeletal muscle 1 hour after exercise (prior to insulin stimulation), and pre and post insulin stimulation at rest and 3 hours after exercise (n = 11). **A**: JNK$^{Thr183/Tyr185}$; **B:** p38 MAPK$^{Thr180/Tyr182}$; **C:** IκBα; **D**: NF-κB p65^{Ser536}; **E**: 4-hydroxynonenal. *p < 0.05, **p < 0.01, ***p < 0.001 and # is significantly different to all time-points in both the rest and exercise trial. Black bars = rest trial. Diagonal line bars = exercise trial. Ex. = High-intensity interval exercise. Ins. = insulin stimulation via the hyperinsulinaemic-euglycaemic clamp.

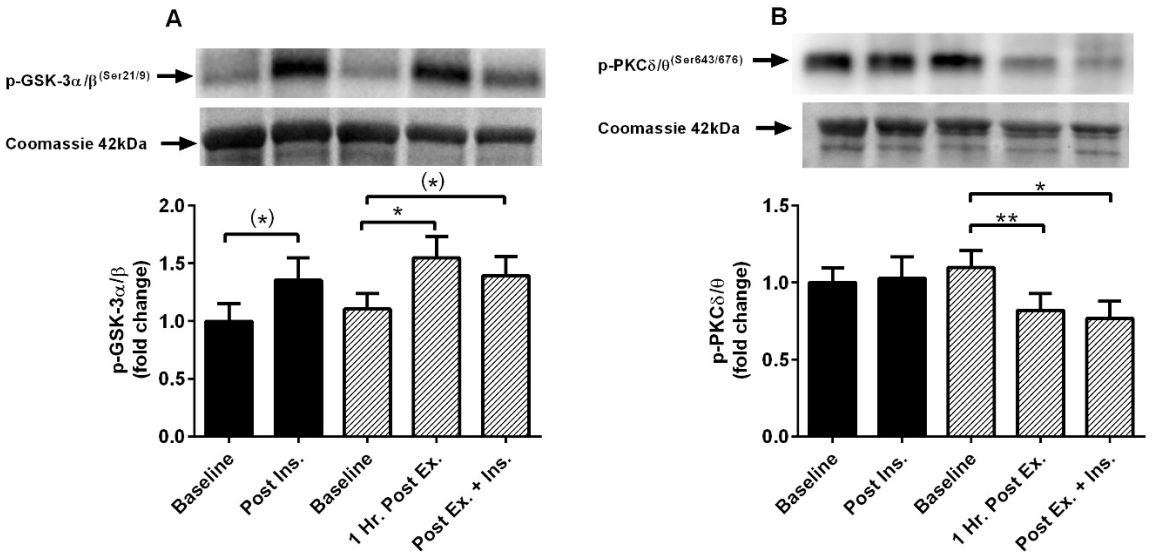

Figure 4.5: Exercise, insulin stimulation, and skeletal muscle glycogen synthase kinase and protein kinase C signalling. Phosphorylation relative to total protein content in skeletal muscle 1 hour after exercise (prior to insulin stimulation), and pre and post insulin stimulation at rest and 3 hours after exercise (n = 11). **A**: GSK-3α/β$^{Ser21/9}$; **B**: PKC δ/θ$^{Ser643/676}$. *p < 0.05 and **p < 0.01 are significantly different. Black bars = rest trial. Diagonal line bars = exercise trial. Ex. = High-intensity interval exercise. Ins. = insulin stimulation via the hyperinsulinaemic-euglycaemic clamp.

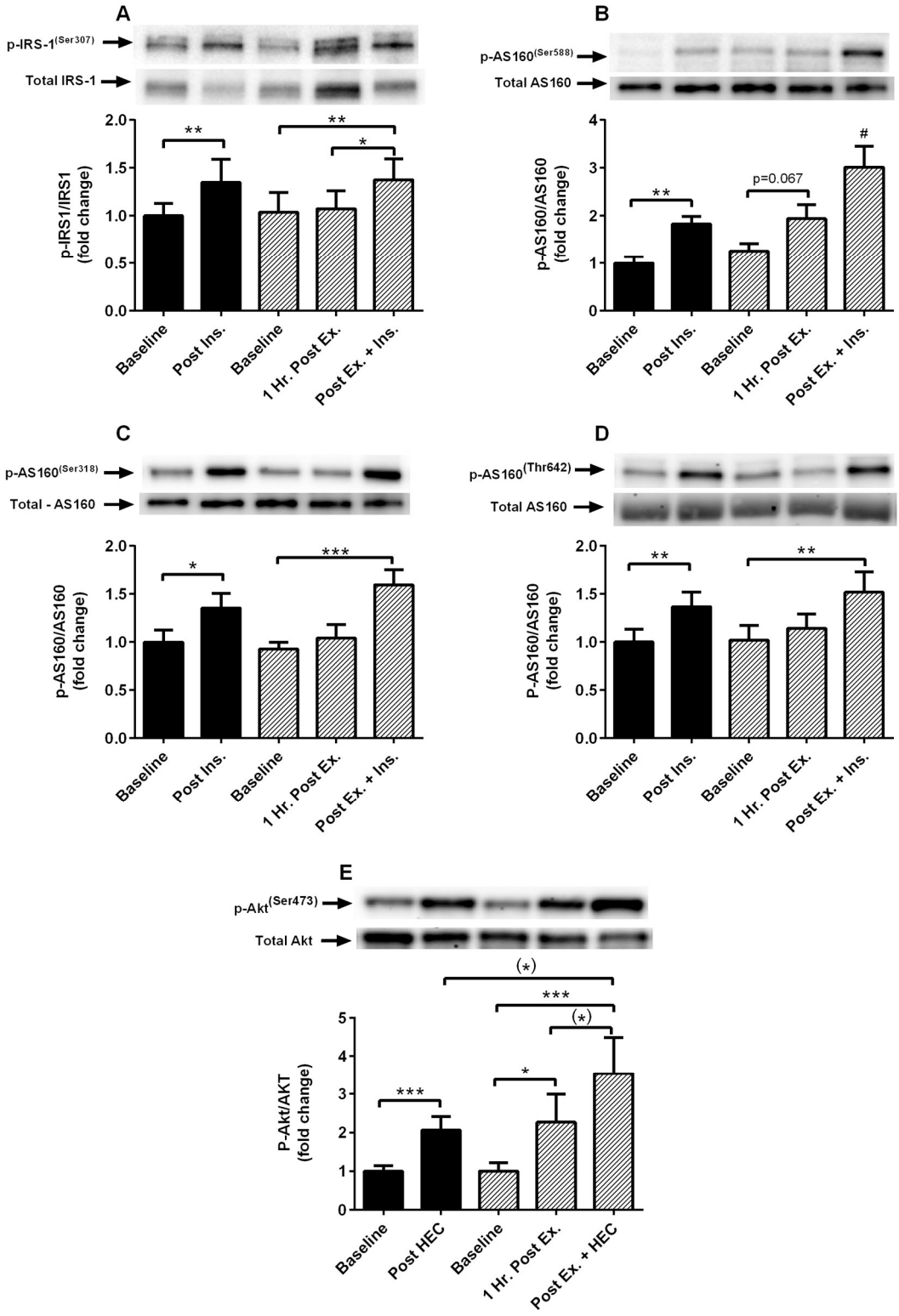

Figure 4.6: Exercise, insulin stimulation and skeletal muscle insulin protein signalling. Phosphorylation relative to total protein content of insulin signalling proteins in skeletal muscle 1 hour after exercise (prior to insulin stimulation), and pre and post insulin stimulation at rest and 3 hours after exercise (n = 11). **A:** IRS-1^{Ser307}; **B:** AS160^{Ser588}; **C**: AS160^{Ser318}; **D**: AS160^{Thr642}; **E:** Akt^{Ser473}. *p < 0.05, **p < 0.01, ***p < 0.001 and # is significantly different to all time-points in both the rest and exercise trial. Black bars = rest trial. Diagonal line bars = exercise trial. Ex. = High-intensity interval exercise. Ins. = insulin stimulation via the hyperinsulinaemic-euglycaemic clamp.

4.5 Discussion.

We report the novel finding that insulin-stimulated phosphorylation of p38 MAPK, NF-κB p65, and JNK occurred to a greater extent after a prior bout of HIIE in obese middle-aged males. Furthermore, increased SAPK signalling coincided with enhanced insulin signalling and whole-body insulin sensitivity, indicating a potential role for SAPK signalling in the post-exercise enhancement of insulin sensitivity. We also reported that insulin stimulation increased plasma antioxidant activity, decreased plasma oxidative stress, and increased 4-HNE in skeletal muscle to a similar extent, irrespective of exercise-induced changes in redox status.

4.5.1 Prior exercise, insulin stimulation, and SAPK and insulin signalling.

We report for the first time that in middle-aged obese males, p38 MAPK, NF-κB p65, and JNK signalling increase with insulin stimulation at rest, and to a greater extent with insulin stimulation after a prior bout of HIIE. Surprisingly, increased p38 MAPK, NF-κB p65, and JNK signalling did not coincide with greater IRS-1^{Ser307} phosphorylation, decreased phosphorylation of Akt^{Ser473} and $AS160^{Ser318,\ Ser588,\ Thr642}$, or impaired insulin sensitivity. Sustained activation of 4-HNE, NF-κB, JNK, p38 MAPK and PKC signalling have been linked to attenuated insulin action and signalling via IRS-1 serine phosphorylation (15, 95, 166, 336). In contrast, the transient activation of SAPK signalling pathways may augment insulin signalling and insulin-stimulated glucose uptake (34, 138, 219, 373, 398, 420). Our findings support redox-sensitive SAPK signalling as a regulator of *in vivo* insulin-stimulated glucose uptake which may, at least in part, contribute to the post-exercise enhancement of insulin sensitivity.

We report similar insulin stimulated phosphorylation of IRS-1^{Ser307} both at rest and after a prior bout of HIIE. Insulin-stimulated phosphorylation of IRS-1^{ser307} may occur due to feedback inhibition of the insulin signalling cascade reported during conditions of hyperinsulinemia (459), which is mediated in part by increased oxidative stress and SAPK signalling (238). Alternatively, IRS-1^{Ser307} phosphorylation may be necessary for insulin-stimulated propagation of the insulin signalling cascade and glucose uptake (92), a physiological process also reliant upon insulin stimulated production of hydrogen peroxide and SAPK

signalling (34, 257, 420). Regardless of the biological role, IRS-1^{Ser307} phosphorylation was similar between rest and exercise trials and therefore unlikely to be contributing to the post-exercise enhancement of insulin sensitivity. These findings support previous reports that post-exercise enhancement of insulin sensitivity likely occurs downstream in the insulin signalling cascade (64, 127).

p38 MAPK can be activated by both insulin and contraction of skeletal muscle, and may play a role in glucose metabolism (398, 420). Current cell culture and animal research is divided with research reporting a role for p38 MAPK in both insulin sensitivity (138, 398) and insulin resistance (98). The discrepancy in findings may be the result of non-specific p38 MAPK inhibitors which can influence metabolism through modification of AKT, GSK-3 and AMPK signalling pathways independent of p38 MAPK (98, 219). However, even when a specific p38 MAPK inhibitor was used phosphorylation/activity of p38 MAPK was either not measured or did not change under the conditions of interest (98). In humans, a recent study found a small but significant reduction (~6%) in post-exercise insulin sensitivity with the infusion of the antioxidant n-acetylcysteine (429). Although p38 MAPK phosphorylation was decreased immediately after exercise with n-acetylcysteine infusion, phosphorylation 3 hours after exercise and a further 2 hours later after insulin stimulation were not different to baseline levels or the placebo intervention (429). In contrast, Thong, et al. (420) reported p38 MAPK phosphorylation in human skeletal muscle to be increased 3 hours after one-legged knee extensor exercise which was further phosphorylated after insulin stimulation. In support of the insulin sensitizing role of p38 MAPK in humans, we measured increased phosphorylation with insulin stimulation at rest, one hour after exercise (prior to insulin stimulation), and the greatest phosphorylation with insulin stimulation after the prior bout of HIIE. The discrepancy in findings between Trewin, et al. (429) and ours may be due to differences in exercise intensity (55 min at 65% $\dot{V}O_{2peak}$ vs. HIIE), the participants' age (young vs. middle-aged), level of physical activity (recreationally active vs. in-active), body composition (BMI 24.8 vs. 33.1 $kg \cdot m^{-2}$) and muscle biopsy sampling times.

The redox-sensitive JNK pathway can be activated by acute exercise and has been suggested to play a role in insulin signalling and glucose metabolism (34, 362, 421). In contrast to the current findings, Castorena, et al. (64) reported enhanced insulin sensitivity 3 hours after exercise in both high fat diet and low fat diet fed rats, despite minimal effect on JNK and IRS-1^{Ser307} phosphorylation. Furthermore, Ropelle, et al. (362) found that a single session of exercise in male rats reversed diet-induced insulin resistance 16 hours later which coincided with decreased JNK, IκB–NF-κB, and IRS-1 serine phosphorylation. In support of the findings of the current study, Berdichevsky, et al. (34) reported that both chronic oxidative stress (1 μM of hydrogen peroxide for 48 hours) and acute oxidative stress (500 μM of hydrogen peroxide for 3 hours) similarly increased JNK phosphorylation in insulin resistant muscle cell lines. Remarkably, chronic oxidative stress increased IRS-1^{Ser307} phosphorylation and insulin resistance whereas acute oxidative stress rescued insulin sensitivity and insulin signalling through redistribution of active cytoplasmic JNK into the nucleus. The effect of JNK and NF-κB protein signalling on the enhancement and/or impairment of insulin sensitivity is likely spatial-temporal sensitive. The current findings support a potential insulin sensitizing role of exercise-induced JNK phosphorylation in overweight, middle-aged males, however further mechanistic research is required to confirm these findings.

In the absence of changes in proximal insulin signalling, exercise-induced SAPK signalling may influence insulin sensitivity through downstream insulin signalling events. Indeed, elevated ROS production caused by glutathione peroxidase-1 knockout mice results in increased phosphorylation of AKT^{Ser473} and enhanced insulin sensitivity 60 mins after treadmill exercise (253). Furthermore, acute hydrogen peroxide exposure (500 μM for 3 hours) in C2C12 myoblasts increases insulin-stimulated glucose uptake alongside increased phosphorylation of JNK, AKT^{Ser473}, AKT^{Thr308} and decreased GSK3-α/β activity (34). In contrast, isolated skeletal muscle of lean Zucker rats incubated in hydrogen peroxide (90 μM for 2 hours) is reported to increase p38 MAPK phosphorylation while concomitantly decreasing AKT^{Ser473} phosphorylation and insulin sensitivity (100). In the present study, we found increased insulin-stimulated SAPK signalling after exercise to coincide with enhanced insulin sensitivity and increased phosphorylation of AS160^{Ser588} and AKT^{Ser473} (242).

Modulation of glycogen synthesis by oxidative stress-induced SAPK signalling is reported to mediate glucose metabolism (34, 100). However, similar insulin-stimulated GSK-3α/β$^{Ser21/9}$ phosphorylation at rest and post-HIIE suggests this as an unlikely pathway for post-exercise enhancement of insulin sensitivity.

Protein kinase C has multiple isoforms which are suggested to play a role in insulin-stimulated glucose uptake (127, 421). The novel PKC δ/θ isoforms are sensitive to both change in redox status and the concentration of the lipid intermediate diacylglycerol (347). PKC δ/θ activation impairs insulin signalling through serine phosphorylation of IRS-1 (164, 244). Interestingly, phosphorylated PKC δ/θ was attenuated 1 hour after HIIE and continued to be so 3 hours later after insulin stimulation. This may be clinically important as obese individuals exhibit elevated levels of plasma free fatty acids (50) and increased PKC activity in response to insulin (244). Indeed, lower insulin-stimulated PKC δ/θ phosphorylation in the exercise trial tended to correlate with higher insulin sensitivity, however phosphorylated IRS-1^{Ser307} was similar between the rest and exercise trial. SAPK signalling, PKC δ/θ activity, and insulin resistance often occur concomitantly (347). In the present study, SAPK signalling increased, PKC δ/θ phosphorylation decreased, and insulin sensitivity was enhanced after HIIE, suggesting independent pathways for PKC δ/θ and SAPK phosphorylation post-HIIE. Thus, PKC δ/θ phosphorylation may be a good candidate for further investigation with respect to post-HIIE enhanced insulin sensitivity in humans.

4.5.2 Redox status and insulin signalling.

The important role of redox biology in promoting and/or attenuating insulin sensitivity is well established in non-human models (421). We provide evidence that insulin stimulation increases plasma antioxidant capacity, decreases plasma oxidative stress, and increases 4-HNE in human skeletal muscle. Furthermore, this insulin stimulated shift in redox status also occurred with insulin stimulation after acute HIIE. Interestingly, the redox shift elicited by exercise (prior to insulin stimulation) had minimal effect on insulin stimulated redox status. Mahadev, et al. (257) reported that insulin stimulation increases hydrogen peroxide via increased NADPH oxidase activity, promoting insulin signalling and glucose uptake in part via decreased protein tyrosine

phosphatase activity. Furthermore, previous research has shown that hydrogen peroxide can both attenuate and/or enhance insulin-stimulated glucose uptake *in-vitro* depending on its concentration (195). It is possible that redox status may be regulated by endogenous antioxidant defences to maintain a redox environment conducive for optimal insulin signalling and glucose uptake. Indeed, alteration of redox homeostasis through exogenous antioxidants in humans are reported to attenuate the benefits of both acute and regular exercise on insulin sensitivity (358, 429). We provide novel in vivo evidence in humans to support the important role of ROS and redox homeostasis in insulin-stimulated glucose uptake (30, 421).

4.5.3 Acute exercise and redox status.

A single session of HIIE increased plasma catalase activity and decreased plasma TBARS and hydrogen peroxide in obese middle-aged males. These findings contradict previous reports of increased systemic oxidative stress in obese individuals (445, 447). The discrepancy in findings are unclear, but may relate to increased antioxidant activity after HIIE, whereas previous studies have reported either no change or decreased antioxidant defence after continuous aerobic exercise (445, 447). Obesity and ageing is associated with higher levels of systemic oxidative stress which over time causes oxidative damage to proteins, lipids and DNA, and the development of numerous pathological conditions (436). Transient shifts in redox homeostasis with regular exercise leads to the upregulation of antioxidant defence, reduces chronic oxidative stress and inflammation, and improves overall metabolic health (258, 358). We provide evidence that regular HIIE may be a beneficial exercise mode for improving redox status and metabolic health in clinical populations.

ROS are beneficial and a necessary requirement for optimal physiological functioning and adaptation to exercise (346). We found 4-HNE protein modification, a marker of oxidative stress, to be increased in skeletal muscle 60 mins after HIIE. This contradictory redox shift between plasma (decreased oxidative stress) and skeletal muscle (increased oxidative stress) may reflect the inability of plasma to accurately reflect skeletal muscle redox status (443). Increased oxidative stress in skeletal muscle likely reflects localized cellular stress associated with muscular contraction and supports the important

signalling role of ROS in the adaptation to exercise (346). Indeed, phosphorylation of redox-sensitive signalling proteins JNK, p38 MAPK and NF-κB were significantly increased 60 mins post-HIIE. Activation of these pathways with exercise is known to promote improvements in redox homeostasis, regulation of energy metabolism, muscle hypertrophy, inflammation, and gene transcription leading to cell proliferation, differentiation and apoptosis (106, 228). Our findings indicate that acute HIIE can transiently shift systemic redox status and activate redox-signalling pathways in skeletal muscle involved in adaptation to exercise in obese middle-aged men.

4.5.4 Limitations.

A potential limitation of the study is the small sample size. However, the present study was adequately powered to detect changes in insulin sensitivity and p38 MAPK phosphorylation in human skeletal muscle (420, 429). Additionally, only a single marker of oxidative stress was used to determine redox status in skeletal muscle. Measurement of multiple redox markers in skeletal muscle would allow for greater interpretation of the influence of skeletal muscle redox status under the conditions of interest. This study is limited to the measurement of muscle and plasma responses to a single session of HIIE and insulin stimulation. Future research would benefit by investigating these findings with respect to subsequent bouts of exercise over a longer period of time. The inclusion of an exercise only control trial would allow for greater understanding of the combined effects of exercise and insulin stimulation on the measured outcomes. Data presented in this study are limited to obese middle-aged males and the specific HIIE protocol used. Further research is required to confirm these findings in other populations and different exercise protocols.

4.5.5 Conclusion.

In summary, we provide evidence that redox status and SAPK signalling are affected by both insulin stimulation and acute HIIE in obese middle-aged males. A prior bout of HIIE elicited greater insulin stimulated JNK, p38 MAPK and NF-κB signalling which coincided with enhanced distal insulin signalling and whole-body insulin sensitivity. These findings support the role of SAPK signalling in glycaemic control and provide potential signalling pathways for the post-

exercise enhancement of insulin sensitivity. Future research is required to explore potential mechanisms.

CHAPTER 5. ACUTE LOW-VOLUME HIGH-INTENSITY INTERVAL EXERCISE AND CONTINUOUS MODERATE-INTENSITY EXERCISE ELICIT A SIMILAR IMPROVEMENT IN 24-HOUR GLYCAEMIC CONTROL IN OVERWEIGHT AND OBESE ADULTS.

Data presented within this chapter has been published in manuscript form and has been adapted for presentation in this thesis:

Parker L., Shaw C.S., Banting L., Levinger I., Hill K.M., McAinch A.J., and Stepto N.K. Acute low-volume high-intensity interval exercise and continuous moderate-intensity exercise elicit a similar improvement in 24-h glycaemic control in overweight and obese adults. Frontiers in Physiology 7: 611, 2017.

This study was in-part funded by an *Australian Government Collaborative Research Network (CRN) grant.*

5.0 General introduction.

In Chapter 4 we explored the effects of a single session of HIIE on post-exercise redox status, SAPK signalling, and insulin sensitivity in obese middle-aged males. Findings indicated that insulin stimulation via the hyperinsulinaemic-euglycaemic clamp significantly increased plasma antioxidant defence and decreased plasma oxidative stress, whereas skeletal muscle oxidative stress, SAPK signalling, and insulin protein signalling increased. Importantly, a prior session of HIIE enhanced insulin stimulated SAPK signalling, insulin protein signalling, and whole-body insulin sensitivity. These findings highlight a potential role for redox-sensitive protein kinase signalling in whole-body insulin sensitivity.

The hyperinsulinaemic-euglycaemic clamp is the current gold standard for measuring insulin sensitivity. However, previous research has suggested that it does not reflect physiological insulin action and glucose dynamics elicited by consumption of a standard meal. Furthermore, laboratory based methods for measuring insulin sensitivity may underestimate functional improvements in glycaemic control. The consumption of a meal results in post-prandial hyperglycaemia and increased systemic oxidative stress, which if in excess, contributes toward the development of insulin resistance, type 2 diabetes, and diabetic complications. A single session of continuous moderate-intensity exercise (CMIE) is reported to improve 24-hour glycaemic control, as measured by continuous glucose monitoring, and attenuates postprandial oxidative stress and glycaemia. However, the influence of low-volume high-intensity interval exercise (LV-HIIE), which consists of considerably less total work and time commitment than CMIE, has yet to be investigated. The aims of Chapter 5 were to compare the effect of LV-HIIE and CMIE on 24-hour glycaemic control and postprandial oxidative stress and glycaemia in overweight and physically inactive individuals.

5.1 Abstract.

Background. Acute exercise reduces postprandial oxidative stress and glycaemia; however, the effects of exercise intensity are unclear. We investigated the effect of acute low-volume high-intensity interval-exercise (LV-HIIE) and continuous moderate-intensity exercise (CMIE) on glycaemic control and oxidative stress in overweight, inactive adults. **Methods.** Twenty-seven adults were randomly allocated to perform a single session of LV-HIIE (9 females, 5 males; age: 30±1 years; BMI: 29±1 $kg \cdot m^{-2}$; M±SEM) or CMIE (8 females, 5 males; age: 30±2.0; BMI: 30±2.0) 1 hour after consumption of a standard breakfast. Plasma redox status, glucose and insulin were measured at baseline, 1 hour after breakfast (prior to exercise), immediately after exercise, 1.5 hours after exercise, and approximately 24 hours after exercise. Continuous glucose monitoring (CGM) was conducted during the 24-hour period before (rest day) and after exercise (exercise day). **Results.** Plasma thiobarbituric acid reactive substances (TBARS; 29 ±13%, $p < 0.01$; mean percent change ±90% confidence limit), hydrogen peroxide (44 ±16%, $p < 0.01$), catalase activity (50 ±16%, $p < 0.01$), and superoxide dismutase activity (21 ±6%, $p < 0.01$) significantly increased 1 hour after breakfast (prior to exercise) compared to baseline. Exercise significantly decreased postprandial glycaemia in whole blood (-6 ±5%, $p < 0.01$), irrespective of the exercise protocol. Only CMIE significantly decreased postprandial TBARS (CMIE: -33 ±8%, $p < 0.01$; LV-HIIE: 11 ±22%, $p = 0.34$) and hydrogen peroxide (CMIE: -25 ±15%, $p = 0.04$; LV-HIIE: 7 ±26%; $p = 0.37$). Acute exercise provided a similar significant improvement in 24-hour average glucose levels (-5 ±2%, $p < 0.01$), hyperglycaemic excursions (-37 ±60%, $p < 0.01$), peak glucose concentrations (-8 ±4%, $p < 0.01$), and the 2-hour postprandial glucose response to dinner (-9 ±4%, $p < 0.01$), irrespective of the exercise protocol. Only LV-HIIE decreased postprandial hydrogen peroxide (LV-HIIE: -20 ±12.8%, $p = 0.03$; CMIE: -3.3±24.1%, $p = 0.73$) 24 hours after exercise. **Conclusions.** Despite elevated postprandial oxidative stress in the hours after exercise compared to CMIE, LV-HIIE attenuated postprandial oxidative stress 24 hours after exercise and elicited a similar improvement in 24-hour glycaemic control in overweight and obese adults.

5.2 Introduction.

Physical inactivity and obesity are major risk factors for impaired glycaemic control, insulin resistance and type 2 diabetes (119, 436). Compared to continuous moderate-intensity exercise (CMIE), high-intensity interval exercise (HIIE) has been shown to elicit comparable and/or greater improvements in glycaemic control (141, 251). Notably, an improvement in glycaemic control can be seen even after a single bout of exercise (144, 247, 437). However, current laboratory based techniques used for assessing glycaemic control, such as the oral glucose tolerance test and the homeostatic model assessment of insulin resistance (HOMA-IR), may not always reflect functional improvements in glycaemic control under free-living conditions (284). Continuous glucose monitoring (CGM) is a reliable and valid method for measuring 24-hour glycaemic status, glycaemic variability, and postprandial responses to meals under free-living conditions (284, 432). A single bout of HIIE can improve 24-hour glycaemic control in obese individuals and patients with type 2 diabetes (144, 247). However, only one study has compared the acute effects of HIIE to CMIE when matched for total workload (247). Consequently, whether shorter duration, lower-volume HIIE (LV-HIIE) provides similar, or greater benefits in 24-hour post-exercise glycaemic control compared to the currently recommended exercise mode of CMIE is unknown.

Oxidation-reduction (redox) status is reported to mediate glycaemic control in both healthy individuals and those with diabetes (421, 473). Oxidative stress occurs as a result of a redox imbalance in favour of excess reactive oxygen species (ROS). This imbalance can result in oxidative modification to DNA, lipids and proteins, playing both a pathological and physiological role in metabolic health (436). Chronic systemic oxidative stress is associated with obesity and physical inactivity, and is linked to the development of insulin resistance and type 2 diabetes (436). Paradoxically, acute exercise also induces a transient state of elevated oxidative stress (120), yet improves insulin sensitivity and glycaemic control (144, 247, 437). While exercise-induced oxidative stress is deemed beneficial and a necessary requirement for optimal tissue functioning and adaptation to physiological stress (346), the effects of exercise-intensity on redox status remain unclear.

Elevated basal and/or postprandial hyperglycaemia elicited through excess nutrient intake, physical inactivity, and/or insulin resistance, is reported to increase systemic oxidative stress through mitochondrial membrane electron leak and the formation of advanced glycation end products (AGEs) (122, 473). Postprandial oxidative stress can last for up to 4 hours after meal consumption and occurs to a greater extent with larger meals that are higher in lipid content (45, 59, 121, 122, 433). In contrast to exercise-induced oxidative stress, excess postprandial systemic oxidative stress contributes to metabolic health complications associated with insulin resistance and type 2 diabetes (119, 122, 433, 473). A single session of low to moderate-intensity exercise in healthy males attenuates the postprandial oxidative stress response to a meal ingested 2 hours before (272) and 24 hours after exercise (414). Furthermore, high-intensity exercise which elicits greater oxidative stress and antioxidant activity compared to low to moderate intensity exercise (120, 323, 380), may also attenuate postprandial oxidative stress (136, 435). Considering the impact of HIIE on postprandial oxidative stress are equivocal (59), and overweight and inactive population's exhibit greater basal and postprandial oxidative stress than healthy controls (119, 433), further research is warranted.

The aim of this study was to test the hypotheses that LV-HIIE would improve 24-hour glycaemic control and postprandial redox status in overweight and obese males and females to a greater extent than CMIE.

5.3 Methodology and procedures.

5.3.1 Participants.

Twenty-seven physically inactive males and females, who were on average overweight (BMI range: 21.4-45.0 $kg \cdot m^{-2}$; 23 out of 27 participants had a BMI >25), volunteered to participate in the study. Participant characteristics are reported in Table 5.1. Females diagnosed with polycystic ovary syndrome (PCOS) were included in the study as they have an intrinsic insulin resistance and are at a 4-fold greater risk of developing type 2 diabetes (63, 407). PCOS diagnosis was self-reported, and supported by personal medical records that adhered to the Rotterdam criteria (115). Participants were sedentary and had not participated in any regular moderate to high levels of physical activity within

the past 3 months. Exclusion for participation included medications known to affect insulin secretion and/or insulin sensitivity; musculoskeletal or other conditions that prevent daily activity; and symptomatic or uncontrolled metabolic or cardiovascular disease. Women with PCOS taking medication (e.g. metformin) were included if medication was stable (> 3 months) and were asked to withdraw medication 48 hours prior to, and throughout the experimental phase of the study. Females were tested in the early follicular phase of the menstrual cycle. Verbal and written explanations about the study were provided prior to obtaining written informed consent. This study was approved by and carried out in accordance with the Victoria University Human Research Ethics Committee for experiments involving humans.

5.3.2 Study design.

Participants were instructed to abstain from physical activity, alcohol and caffeine consumption (48 hours) prior to, and throughout, the 4 consecutive days of the experimental period. In brief, day one involved insertion of the CGM and participant familiarization; Day two was a rest day with dietary control; Day three was an exercise day (LV-HIIE or CMIE) with dietary control; Day four involved the removal of the CGM and a 24-hour post-exercise blood sample (Figure 5.1).

Table 5.1: Descriptive characteristics of participants in the LV-HIIE and CMIE protocol group

	LV-HIIE	CMIE	p value
Participants	14	13	
Males	5	5	
Females	9	8	
Females with PCOS	6	5	
Age (years)	30 ± 1	30. ± 2	0.96
Height (cm)	169.5 ± 2.7	166.4 ± 2.3	0.40
Weight	84.1 ± 5.1	83.3 ± 5.6	0.92
BMI ($kg \cdot m^{-2}$)	29.2 ± 1.4	30.0 ± 1.8	0.70
BMI >25 $kg \cdot m^{-2}$	4 males 8 females	4 males 7 females	
Waist to hip ratio	0.82 ± 0.02	0.84 ± 0.03	0.85
Systolic blood pressure (mm Hg)	122 ± 3	117 ± 3	0.20
Diastolic blood pressure (mm Hg)	85 ± 3	78 ± 3	0.07
W_{max} (Watts)	175 ± 19	170 ± 14	0.82
Max heart rate (BPM)	186 ± 3	180 ± 4	0.22
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	28.7 ± 2.2	28.8 ± 1.9	0.98
Total exercise session work (kJ)	147 ± 13	191 ± 15*	0.04
Total exercise session duration (min)	24	38*	< 0.01
HOMA2-IR	1.4 ± 0.1	1.4 ± 0.2	0.78
Fasting glucose (mmol/l)	4.5 ± 0.1	5.0 ± 0.3	0.12
Fasting insulin (pmol/l)	88 ± 7	84 ± 12	0.78

Values are mean ± SEM. * $p < 0.05$ compared to LV-HIIE. **LV-HIIE:** low-volume high-intensity interval exercise. **CMIE:** continuous moderate-intensity exercise. **PCOS:** polycystic ovary syndrome. **HOMA2-IR:** homeostatic model assessment of insulin resistance version 2. **BMI:** body mass index.

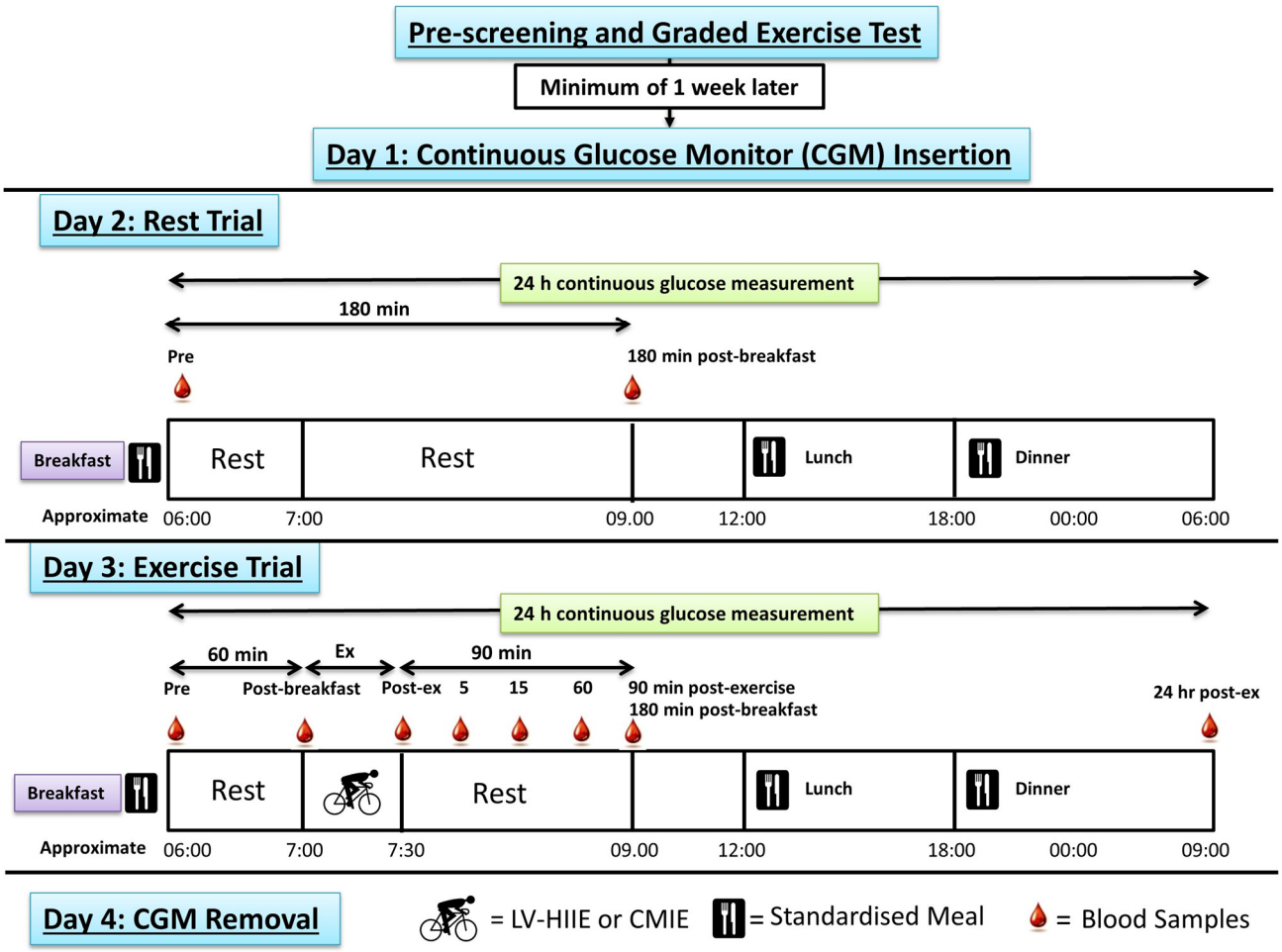

Figure 5.1: Detailed schematic of the research methodology and procedures. Participants were pre-screened and randomized into either LV-HIIE or CMIE exercise groups. Participants than underwent 4 consecutive days of testing. Day 1 consisted of CGM insertion and instruction of its appropriate use. Day 2: consisted of a rest non-exercise day. Day 3 was identical to day 2 with the addition of a single session of either LV-HIIE or CMIE performed 1 hour after breakfast consumption. Day 4 consisted of removal of the CGM and a 24-hour post-exercise blood sample. Participants were provided a standard breakfast, lunch, dinner and snacks, to eat throughout the 4-day testing period. Meals were consumed at the same time of day over the testing period. Venous blood samples were taken at the time points indicated in the figure. **CMIE:** Continuous moderate-intensity exercise. **LV-HIIE:** low-volume high-intensity interval exercise. **CGM:** continuous glucose monitor.

5.3.3 Screening and preliminary testing.

Participants were pre-screened via a medical history and risk assessment questionnaire. Eligible participants underwent body composition analysis, and a graded exercise test (GXT) on a cycle ergometer (Lode Excalibur Sport) to measure aerobic capacity (VO_{2max}) and maximal power output (W_{max}). Expired gases were collected and analysed via a metabolic system (Moxus Modular VO2 System). The GXT protocol consisted of 3 minutes cycling (60 RPM) at 50 watts, increasing by 25 watts every 3 minutes for the first three stages, and then increasing every 1 minute thereafter. Participants cycled until they were unable

to maintain 50 revolutions per minute. The maximum wattage obtained (W_{max}) during the exercise test was used to calculate the workload for the LV-HIIE or CMIE in the main experiment. Heart rate (HR) was recorded using a 12-lead electrocardiograph system along with the 6-20 Borg scale rating of perceived exertion (RPE).

One to three weeks after completing the pre-screening session participants were randomized into either the LV-HIIE or CMIE exercise interventions stratified by sex and BMI using a minimisation randomisation model (424). Using this method of randomisation, participants were sometimes allocated their group (HIIE or CMIE) not purely by chance, but by determining in which group inclusion of the participant would minimise any differences in the confounding factors of BMI and sex. This procedure is called a minimisation randomisation model, and is deemed an acceptable and often preferred method of randomisation in clinical trials (424).

5.3.4 Experimental phase.

Experimental day 1. Participants reported to the laboratory for fitting of the CGM (Guardian® Real-Time, Medtronic, USA) and detailed instructions of its use (432). Calibration of the CGM was performed three times per day (morning, lunchtime and evening), and performed at least 2 hours after the participants last meal. Participants were instructed to consume the food provided and refrain from any physical activity not prescribed by the researchers. Prior to leaving the laboratory, participants were given a standardized meal (dinner) to take home and consume at their normal meal time.

Experimental day 2. Participants reported to the laboratory in the morning after an overnight fast and consumed a standardized breakfast. Immediately following breakfast participants rested in the laboratory for a total of three hours. Blood samples were taken at baseline (prior to breakfast) and 3 hours after breakfast. Participants were provided standardized meals for lunch, dinner and snacks to take home and eat at their preferred meal times, and were instructed to detail all food consumption and physical activity in the provided log books.

Experimental day 3. Participants again reported to the laboratory in the morning after an overnight fast. As per the previous rest day, participants

consumed the standardized breakfast. One hour later, participants performed a single session of either the LV-HIIE or CMIE protocol. The participants then rested in the laboratory for 90 minutes. The standardized meals were again provided including the final day's breakfast (day 4), to be consumed at the same time of day as per the previous two days. Blood samples were taken at baseline, pre-exercise (1 hour after breakfast), and immediately after exercise, 5, 15, 60 and 90 minutes after exercise (~3 hours after breakfast).

Experimental day 4. Participants arrived in the laboratory ~3 hours after consumption of their standard breakfast and the CGM was removed. A blood sample was taken approximately 3 hours after breakfast on the day after LV-HIIE and CMIE (approximately 24 hours after exercise) for postprandial plasma redox status analysis. It must be noted that variations in participant's availability to attend the laboratory for this blood sample (181±10 minutes after breakfast in the LV-HIIE group, and 170±10 minutes in the CMIE group) limit interpretation of this sample.

5.3.5 Exercise protocols.

The LV-HIIE protocol consisted of a five-minute warm-up at 50% of the participants W_{max} obtained during the GXT. Following the warm-up, participants performed 8 x 1-minute cycling bouts at 100% of W_{max} (175±19 Watts), interspersed with 1-minute active recovery periods cycling at 50 watts. A 3-minute cool down was then performed at 50% of W_{max}. The total workout session duration was 24 minutes. The CMIE session consisted of 38 ± 1 minutes cycling at 50% of the participants W_{max} (79±9 Watts). Total work performed on the cycle ergometer during the LV-HIIE and CMIE sessions are reported in Table 5.1.

5.3.6 Dietary control.

Daily energy and macronutrient intake for the standardized meals were based on sex, height and weight, and consisted of approximately 55% carbohydrate, 30% fat and 15% protein, adhering with the Australian and New Zealand dietary targets (89). With the exception of necessary dietary substitutions (vegetarian, halal etc.) breakfast consisted of Kellogg's® Corn Flakes and Kellogg's® All-Bran®, honey and full cream milk; lunch consisted of canned tuna, tomato,

lettuce and carrot roll/s; dinner consisted of sausages, cooked white rice, sweet potato, and mixed frozen vegetables; and snacks consisted of a muffin, banana and yoghurt. To ensure consistency throughout the study, participants were instructed to eat breakfast, lunch and dinner at the same time of day over the 4-day experimental period, and were instructed to log all physical activity (time of day, exercise mode, duration and intensity) and food consumed (time of day, type and quantity of food eaten) in the provided log books.

5.3.7 Blood sampling.

Venous blood was collected from an antecubital vein via an intravenous cannula and collection tube and kept patent with 0.9% sterile saline. Blood was collected in appropriate tubes and immediately centrifuged at 3,500 rpm for 15 min at 4 °C, the plasma was aliquoted and stored at -80 °C until analysed.

5.3.8 Biochemical analysis.

Whole blood lactate and glucose were analysed using an automated analysis system (YSI 2300 STAT Plus™ Glucose & Lactate Analyser). Plasma insulin levels were determined via an automatic gamma counter (2470 WIZARD 2, PerkinElmer Life Sciences, Boston, MA) in duplicate using radioimmunoassay in accordance with the manufacturer's instructions (HI-14K kit, Millipore). Insulin resistance was estimated using the homeostatic model assessment (version 2) for insulin resistance (HOMA2-IR) using the Oxford Diabetes Trials Unit calculator (https://www.dtu.ox.ac.uk/homacalculator; University of Oxford, UK).

5.3.9 Plasma redox status analysis.

Plasma thiobarbituric acid reactive substances (TBARS; Cayman), catalase activity (Cayman), superoxide dismutase activity (SOD; Cayman) and hydrogen peroxide (Amplex UltraRed assay, Molecular Probes) were determined on a spectrophotometer (xMark microplate spectrophotometer, Bio-Rad Laboratories) in duplicate as per the manufacturer's instructions. Intra-assay coefficients of variation were determined and averaged from each duplicate for all participants and resulted in a coefficient of variation of 2%, 3%, 3% and 2% for TBARS, SOD, Catalase and hydrogen peroxide, respectively. Inter-assay coefficients of variation for assay standards between each 96 well plate were

averaged and resulted in a coefficient of variation of 1%, 4%, 4%, 1%, for TBARS, SOD, catalase and hydrogen peroxide, respectively.

5.3.10 Continuous glucose monitor analysis.

Five-minute glucose values recorded by the CGM over the 4-day intervention were exported. Meal-times were cross-checked with participants' diet log books and data were checked for missing values and/or abnormal readings. The 24-hour period prior to the onset of exercise (rest day) and the 24-hour period immediately after the onset of exercise (exercise day), were used to compare 24-hour CGM determined glycaemic control. For consistency, missing data points were handled as per previous publications using CGM technology (247). Briefly, if less than 3 consecutive five-minute periods were missing the average of the glucose value before and after were inserted. If greater than 3 consecutive five-minute periods were missing over the 24-hour period of comparison, then both the rest and exercise days were adjusted to omit these values. Continuous glucose monitoring data was corrupted for 1 participant in the LV-HIIE, and was excluded from the CGM comparisons.

Average glucose values, peak glucose concentration, total area under the curve (AUC) and incremental AUC (iAUC), were calculated for the 24-hour rest day and exercise day period, and the 2-hour postprandial period following consumption of breakfast, lunch and dinner. The 24-hour glycaemic variability measurements of the standard deviation (SD) of the mean glycaemia, the mean amplitude of glycaemic excursions (MAGE), and the percentage coefficient of variation (% CV), were calculated using the GlyCulator windows software package as previously described (91). The percentage of time spent with hyperglycaemia (above 7 mmol/l) during the 24-hour time period was also determined.

5.3.11 Statistical analysis and sample size calculation.

Data were checked for normality and analysed using Predictive Analytics Software (PASW v20, SPSS Inc.). Comparisons of means for the CGM data were examined using a two-factor repeated measures analysis of variance (ANOVA) with trial day (rest or exercise day) as the within-subject factor and group (LV-HIIE or CMIE) as a between-subjects factor. Comparison of multiple

means for biochemical analysis, heart rate and RPE, on the exercise day were analysed using a two-factor repeated measures ANOVA with time as the within-subjects factor and group (LV-HIIE and CMIE) as the between-subjects factor. A three-factor repeated measures ANOVA was conducted to investigate the effect of exercise on postprandial oxidative stress with time (baseline and 3 hours postprandial) and day (rest day and exercise day) as within-subject factors and group (LV-HIIE and CMIE) as the between-subjects factor. Post hoc analysis of significant interaction and main effects were performed using Fisher's protected LSD test. All data are reported as mean ± standard error of mean (SEM) and all statistical analysis were conducted at the 95% level of significance ($p \leq 0.05$). Trends were reported when p-values were greater than 0.05 and less than 0.1.

A priori calculations (G*Power 3.1. 9) indicated that with an alpha set at 0.05 and power at 0.8, a sample size of nine in each group was required to detect a significant ($p < 0.05$) improvement in dinner postprandial glycemia after an acute morning session of exercise. This calculation was based on a previously reported effect size of 1.13 (mean improvement of 52 ± 46 mmol/l*2 hr; n = 9) (247). Thus, the study was adequately powered for the main outcome measure of post-exercise enhancement of postprandial glycemia.

5.4 Results.

Measures of fasting glucose, fasting insulin, and HOMA2-IR were not statistically different between participants with and without PCOS, or between PCOS participants in the LV-HIIE and CMIE groups ($p > 0.05$; data not shown), as such data were pooled together for analysis. Baseline measurements for CGM and biochemical data, and physiological data measured in the GXT, were not significantly different ($p > 0.05$) between LV-HIIE and CMIE groups (Table 5.1).

5.4.1 Physiological response to exercise.

HR and RPE were significantly higher during LV-HIIE compared to CMIE (Figure 5.2). Total work (kJ) and exercise duration (minutes) in the CMIE group were significantly greater than LV-HIIE (Table 5.1).

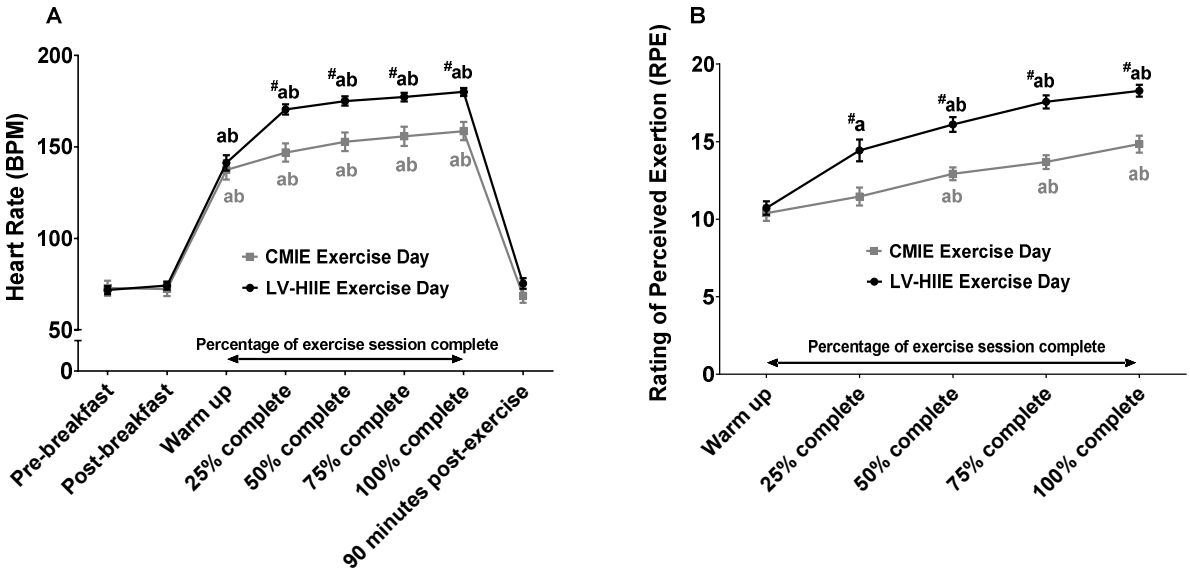

Figure 5.2: Rating of perceived exertion and heart rate response to LV-HIIE and CMIE. Heart rate **(A)** and rating of perceived exertion **(B)** during exercise for both LV-HIIE and CMIE. a - $p < 0.05$ compared to pre-breakfast or warm up; b - $p<0.05$ compared to 1-hour post-breakfast or repetition 2 (25% of CMIE); # - $p < 0.05$ compared to the corresponding time point in the CMIE group. **CMIE:** continuous moderate intensity exercise. **LV-HIIE**: low-volume high-intensity interval exercise. Values are means ± SEM.

5.4.2 Continuous glucose monitor data.

Average glucose concentration, glucose AUC, peak glucose concentration, and the percentage of the day spent with glucose values above 7 mmol/l were significantly lower during the 24-hour period after exercise compared to the rest day (all $p < 0.05$) with no differences between LV-HIIE and CMIE (all $p > 0.05$; Table 5.2). Exercise significantly decreased dinner 2-hour average glucose concentration, peak glucose concentration, AUC and iAUC (all $p < 0.05$; Table 5.2). Exercise significantly decreased breakfast total and iAUC (both $p < 0.05$), whereas a significant interaction effect ($p = 0.04$) and subsequent post hoc analysis indicated that only CMIE decreased breakfast 2-hour average glucose concentration ($p < 0.01$; Table 5.2). The glycaemic variability measurements of MAGE, SD, and % CV were not significantly different between trial days or groups (all $p > 0.05$; Table 5.2). Representative graphs of the mean difference between the rest day and exercise day over the 24-hour period, and the CGM postprandial breakfast response, are reported in Figure 5.3.

5.4.3 Exercise day biochemical analysis.

Blood glucose, plasma insulin, hydrogen peroxide, TBARS, catalase activity, and SOD activity were not significantly different between groups at baseline (all $p > 0.05$). Significant interaction effects were detected for plasma insulin ($p = 0.01$), glucose ($p = 0.01$), lactate ($p < 0.01$), hydrogen peroxide ($p = 0.05$), and TBARS ($p < 0.01$). Post-hoc analysis revealed that plasma insulin was significantly higher 1 hour after breakfast compared to baseline for both the LV-HIIE and CMIE group (Figure 5.4). Immediately, and 1.5 hours after CMIE, insulin levels returned to baseline. This was not evident after LV-HIIE where insulin levels remained significantly elevated. Blood glucose levels were significantly lower than baseline immediately after exercise with CMIE, and significantly lower than baseline 1.5 hours after exercise after both CMIE and LV-HIIE (Figure 5.4). Blood lactate was significantly higher at all time points after LV-HIIE compared to CMIE (Figure 5.4).

Plasma hydrogen peroxide, TBARS, catalase activity, and SOD activity were significantly higher 1 hour after breakfast (Figure 5.5). SOD activity and catalase activity remained significantly elevated during the recovery period after both CMIE and LV-HIIE. On the other hand, post-hoc analysis of hydrogen peroxide and TBARS remained significantly elevated during the recovery period after LV-HIIE only (Figure 5.5).

Table 5.2: Analysis of continuous glucose monitoring measurements during the rest and exercise day for both LV-HIIE and CMIE trials.

Variable	LV-HIIE		CMIE	
	Rest day	Exercise day	Rest day	Exercise day
24hr measurements (mmol/l)				
Average blood glucose	4.7±0.1	4.5±0.1*	5.3±0.3	5.1±0.3*
Total AUC	6438±192	6077±214*	7174±506	6847±475*
Peak glucose concentration	7.2±0.3	6.5±0.3*	7.9±0.6	7.2±2.0*
2hr PPG (mmol/l)				
Breakfast	5.1±0.2	5.3±0.3	6.1±0.5	5.4±0.4*
Breakfast (1st hour)	5.4±0.2	5.4±0.2	6.2±0.4	5.8±0.4
Breakfast (2nd hour)	4.9±0.2	4.5±0.2*	5.9±0.6	5.0±0.4*
Lunch	5.0±0.2	5.1±0.1	5.9±0.3	5.7±0.3
Dinner	5.2±0.3	4.6±0.2*	6.2±0.4	5.7±0.3*
2hr PPP (mmol/l)				
Breakfast	6.1±0.2	6.3±0.3	7.4±0.7	6.8±0.6
Lunch	5.7±0.2	5.9±0.3	7.1±0.4	6.8±0.5
Dinner	5.9±0.4	5.2±0.3*	7.1±0.5	6.5±0.4*
2hr iPPP (mmol/l)				
Breakfast	1.6±0.2	1.7±0.3	2.4±0.4	1.9±0.3
Lunch	1.5±0.2	1.6±0.3	2.5±0.3	2.0±0.4
Dinner	1.5±0.3	1.1±0.2*	2.1±0.3	1.8±0.3*
2hr AUC (mmol/l·2hr)				
Breakfast	617±25	609±29*	730±63	655±49*
Lunch	601±25	610±16	711±36	709±32
Dinner	629±34	569±23*	748±53	687±41*
2hr iAUC (mmol/l·2hr)				
Breakfast	88.6±11.9	80.5±18.6*	105.0 ±13.7	71.3±14.0*
Lunch	107.7±16.2	93.3±11.7	173.1±20.8	153±27.7
Dinner	107.2±18.0	75.9±20.4*	154.3±30	129.4±26.9*
Glycaemic variability				
MAGE	2.2±0.2	2.2±0.2	2.6±0.3	2.2±0.3
SD	0.7±0.1	0.7±0.1	0.8±0.1	0.8±0.1
%CV	15.1±1.2	15.7±1.6	15.58±1.3	14.58±1.7
% of 24hr day at >7 mmol/l$^{\delta}$	2.7±0.9	0.2±0.2*	11.4±6.3	8.5±5.3*

Values are mean±SEM (n = 13 in both LV-HIIE and CMIE groups for CGM data). * - $p < 0.05$ are significantly different to the rest day. δ - 7 out of 13 participants in the LV-HIIE group and 10 out of 13 in the CMIE group exhibited glucose values higher than 7 mmol/L. **LV-HIIE**: low-volume high-intensity interval exercise. **CMIE:** continuous moderate-intensity exercise. **MAGE**: 24-hour mean amplitude of glycaemic excursion. **SD:** 24-hour glycaemic standard deviation. **%CV:** 24-hour glucose percentage coefficient of variation. **PPG**: postprandial glucose. **PPP:** peak postprandial glucose. **iPPP:** incremental peak postprandial glucose. **AUC:** area under the glucose curve. **iAUC:** incremental area under the glucose curve.

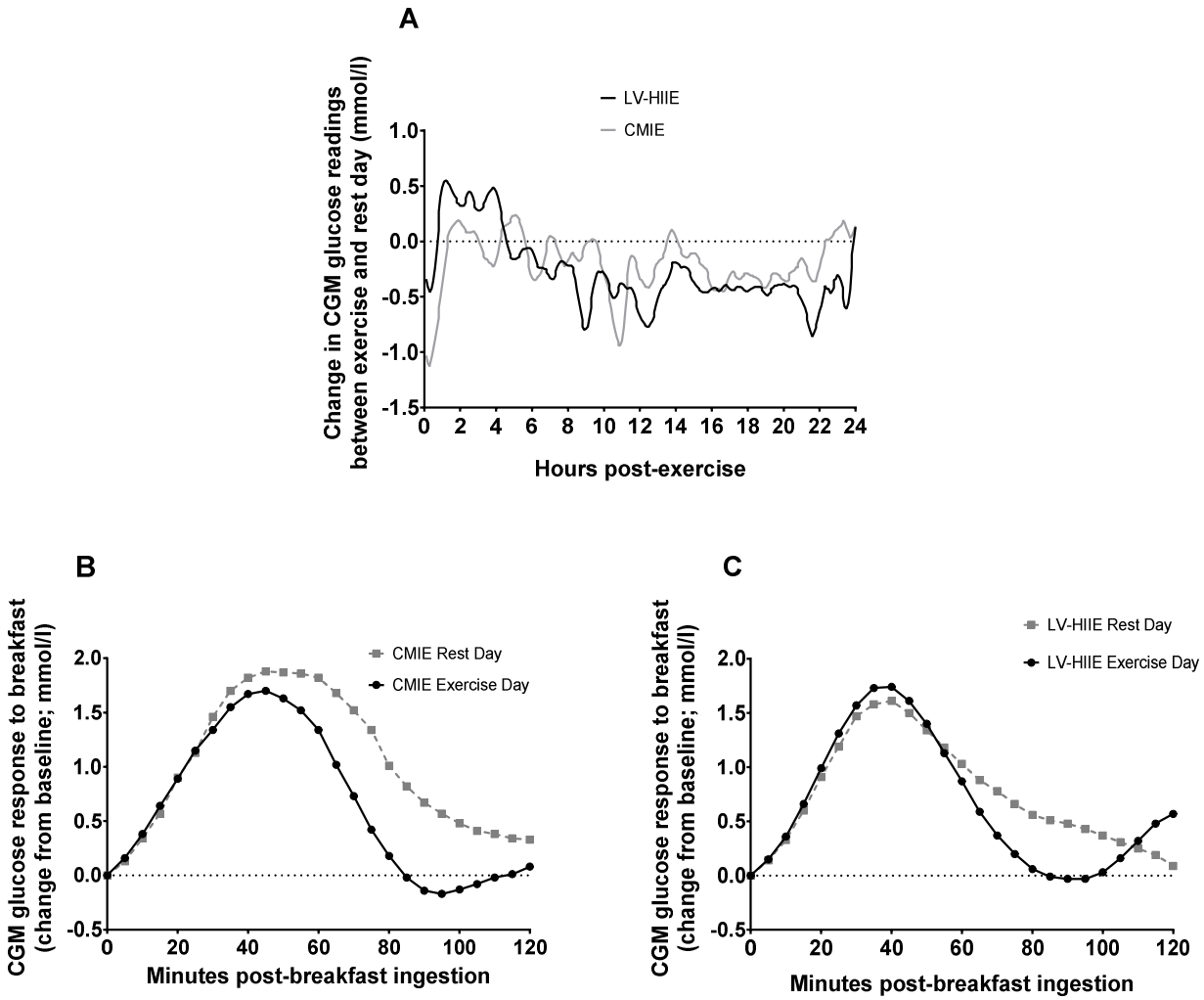

Figure 5.3: Continuous glucose monitor traces for 24-hour glucose and postprandial breakfast. Change in average CGM glucose readings (mmol/l) from rest to exercise day over the 24-hour period **(A)**. Average postprandial CGM glucose readings (change from baseline; mmol/l) on the rest day and when CMIE **(B)** or LV-HIIE **(C)** were performed 1 hour after breakfast consumption. **CMIE**: continuous moderate intensity exercise. **LV-HIIE:** low-volume high-intensity interval exercise. **CGM:** continuous glucose monitor.

5.4.4 The effect of exercise on 3-hour postprandial oxidative stress.

A significant interaction (group*time*day) effect ($p = 0.03$) was detected for hydrogen peroxide. Post-hoc analysis indicated that compared to baseline hydrogen peroxide was significantly elevated 3 hours after breakfast on the rest day with both CMIE and LV-HIIE groups ($p < 0.05$; Table 5.3). Furthermore, 3-hour postprandial hydrogen peroxide was elevated to a greater extent ($p < 0.05$) on the exercise day compared to the rest day with LV-HIIE but not CMIE (Table 5.3). There was a trend towards significance ($p = 0.06$) for an interaction effect (group*time*day) for TBARS (Table 5.3). Post-hoc analysis indicated that

compared to the rest day there was a tendency for greater TBARS at 3 hours postprandial compared to the exercise day with LV-HIIE ($p = 0.08$) and lower TBARS on the exercise day with CMIE ($p = 0.07$). Furthermore, TBARS was significantly greater on the exercise day at 3 hours postprandial with LV-HIIE compared to CMIE ($p < 0.05$; Table 5.3). A main effect of time ($p < 0.01$) indicated significantly greater catalase activity at 3 hours postprandial compared to baseline irrespective of exercise group or day (Table 5.3). A significant interaction (group*time*day; $p = 0.043$) effect was detected for SOD activity. Post-hoc analysis indicated significantly greater SOD activity at 3 hours postprandial compared to baseline with both CMIE and LV-HIIE groups on the rest day, and with LV-HIIE on the exercise day (all $p < 0.05$). Compared to the rest day, there was a trend ($p = 0.051$) for decreased SOD activity at 3 hours postprandial on the exercise day with CMIE.

Comparison of the change (3-hour postprandial value minus the baseline value) between the rest day and exercise days indicated that LV-HIIE elicited a greater increase in hydrogen peroxide ($p = 0.03$), TBARS ($p = 0.06$), and SOD activity ($p = 0.04$) on the exercise day compared to CMIE.

Twenty-four hours after LV-HIIE, postprandial oxidative stress and antioxidant activity were lower compared to the rest day (hydrogen peroxide and catalase activity) and the exercise day (TBARS, Hydrogen peroxide, SOD and catalase activity; Figure 5.6). In contrast, 24 hours after CMIE, both TBARS and SOD activity were higher compared to the exercise day, whereas catalase activity was lower compared to both the rest and exercise day (Figure 5.6).

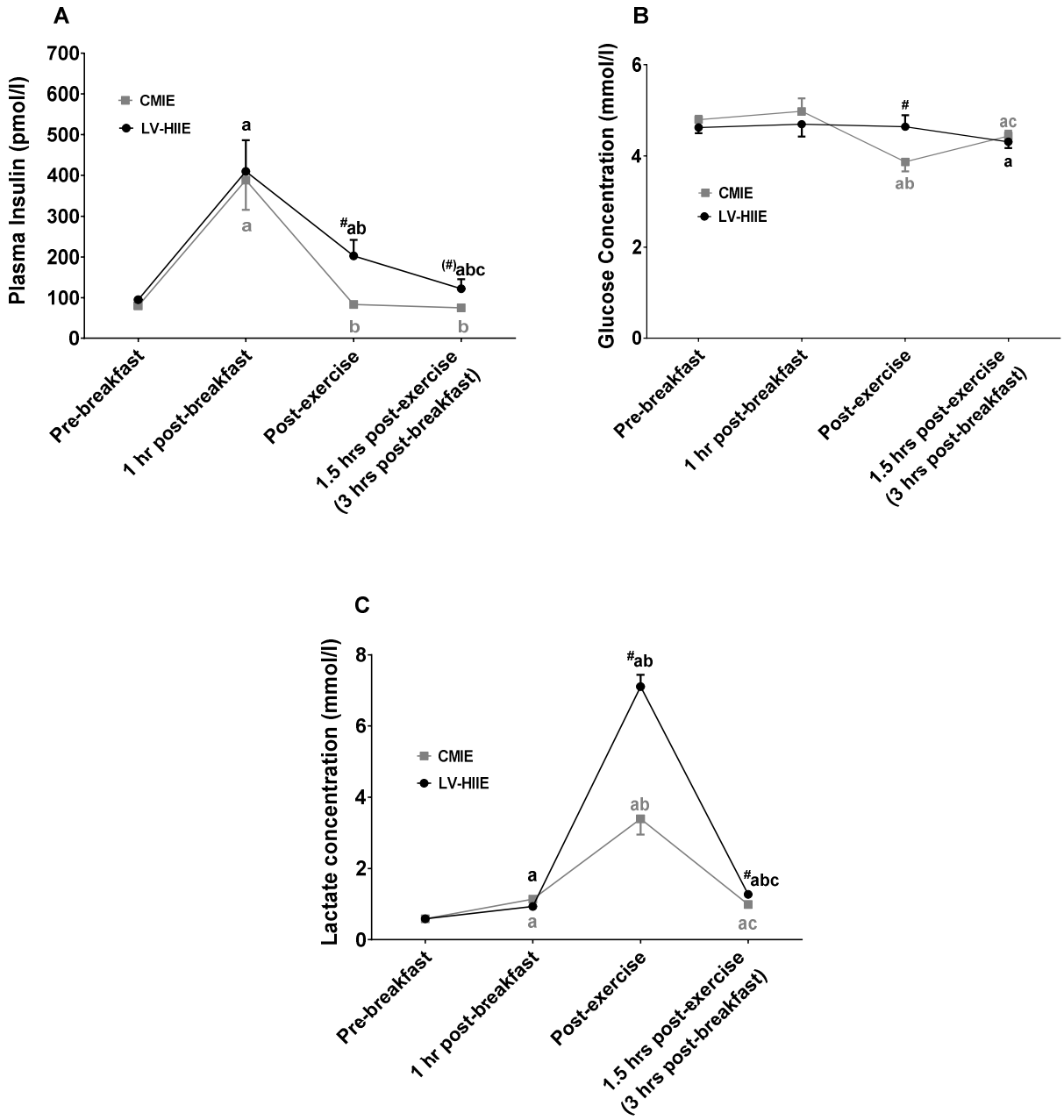

Figure 5.4: Biochemical blood and plasma response to breakfast and exercise. The postprandial and post-exercise response of plasma insulin **(A)**, blood glucose **(B)**, and blood lactate **(C)**. a - $p<0.05$ compared to baseline; b - $p < 0.05$ compared to 1 hour post-breakfast; c - $p < 0.05$ compared to post-exercise; # - $p < 0.05$ compared to the equivalent time point in the CMIE group. Symbols in parenthesis indicate $p < 0.1$. **CMIE:** continuous moderate intensity exercise. **LV-HIIE:** low-volume high-intensity interval exercise. Values are means ± SEM.

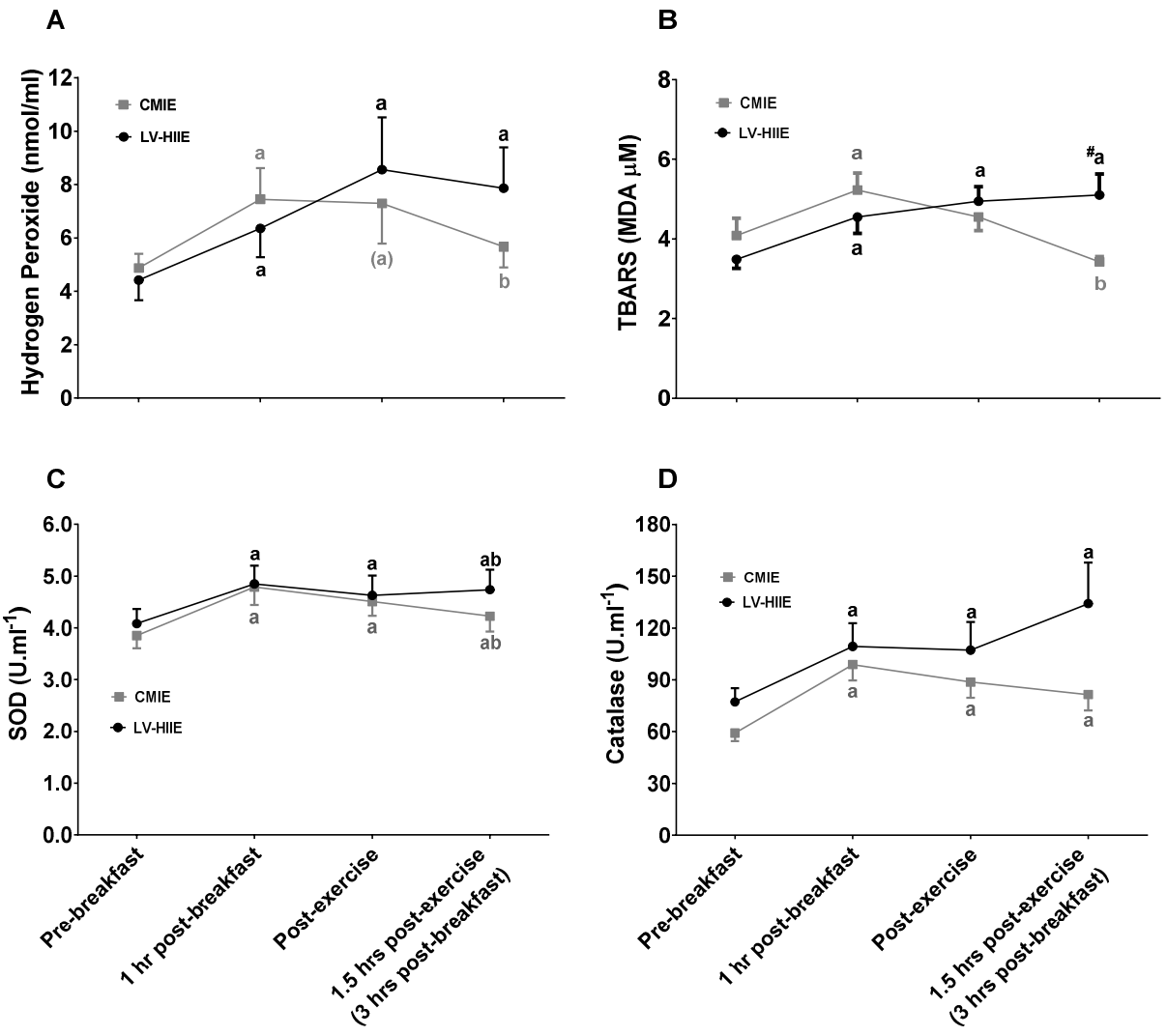

Figure 5.5: Plasma redox status response to breakfast and exercise. Plasma redox status response to ingestion of a standard breakfast and when either LV-HIIE or CMIE was performed 1 hour after breakfast. The postprandial and post-exercise response of plasma hydrogen peroxide **(A)**, **TBARS (B)**, SOD activity **(C)**, and catalase activity **(D)**. a - $p < 0.05$ compared to baseline; b - $p < 0.05$ compared to 1 hour post-breakfast; # - $p < 0.05$ compared to the equivalent time point in the CMIE group. Symbols in parenthesis indicate $p < 0.1$. **CMIE:** continuous moderate intensity exercise. **LV-HIIE:** low-volume high-intensity interval exercise. **SOD:** superoxide dismutase. **TBARS:** thiobarbituric acid reactive substances. Values are means ± SEM.

Table 5.3: The effect of exercise intensity and volume on plasma postprandial oxidative stress.

	Rest Day			Exercise Day			
	Baseline	3 hours postprandial	Change on rest day (Cohens d)	Baseline	3 hours postprandial	Change on exercise day (Cohens d)	Change between exercise and rest day (Cohens d)
Hydrogen peroxide (nmol.ml^{-1})							
LV-HIIE	4.5±0.8	5.6±1.0*	1.1±0.5 (0.37)	4.4±0.7	7.9±1.5*†	3.4±1.3 (1.46)	2.3±1.1# (1.44)
CMIE	5.4±0.8	6.8±1.0*	1.4±0.4 (0.46)	4.9±0.5	5.7±0.7	0.8±0.6 (0.34)	-0.6±0.4 (-0.36)
TBARS (MDA µM)							
LV-HIIE	3.2±0.2	4.0±0.3	0.8±0.3 (0.68)	3.5±0.2	5.1±0.5*#(†)	1.6±0.6 (1.30)	0.8±0.8(#) (0.52)
CMIE	4.1±0.4	4.6±0.4	0.5±0.5 (0.44)	4.1±0.4	3.4±0.1(†)	-0.7±0.4 (-0.53)	-1.2±0.5 (-0.77)
Catalase (U.ml^{-1})							
LV-HIIE	32.6±2.4	42.9±5.9*	10.3±5.1 (1.24)	32.8±3.3	57±9.8*	24.2±8.3 (2.25)	13.9±8.4 (0.73)
CMIE	25.2±1.6	39.2±6.0*	14.0±5.2 (1.69)	25.1±1.9	34.6±3.8*	9.5±3.9 (0.88)	-4.5±6.0 (-0.24)
SOD (U.ml^{-1})							
LV-HIIE	4.1±0.3	4.5±0.3*	0.4±0.1 (0.44)	4.1±0.3	4.7±0.4*	0.7±0.3 (0.69)	0.2±0.2# (0.50)
CMIE	3.9±0.3	4.6±0.3*	0.7±0.1 (0.76)	3.9±0.2	4.2±0.3(†)	0.4±0.2 (0.39)	-0.3±0.2 (-0.68)

Values are mean±SEM. **LV-HIIE:** low-volume high-intensity interval exercise. **CMIE**: continuous moderate-intensity exercise. **SOD:** superoxide dismutase. **TBARS:** thiobarbituric acid reactive substances. * - $p < 0.05$ compared to baseline; † - $p < 0.05$ compared to rest day; # - $p < 0.05$ compared to CMIE; symbols in parenthesis indicate $p < 0.1$.

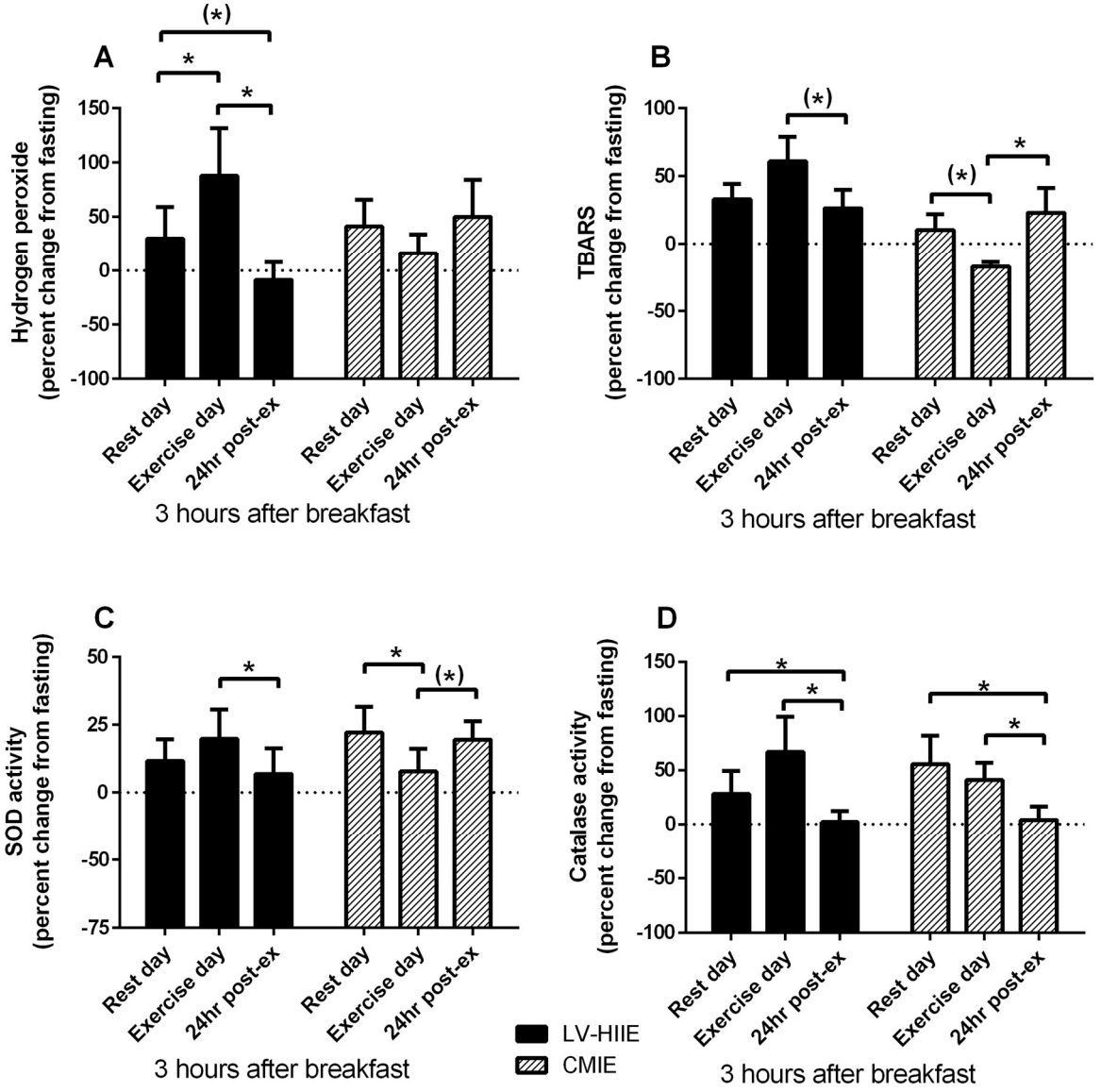

Figure 5.6: The effect of exercise-intensity on 3-hour postprandial plasma oxidative stress and antioxidant activity. Percent change from fasting baseline levels and 3-hour postprandial hydrogen peroxide **(A)**, TBARS **(B)**, SOD activity **(C)**, and catalase activity **(D),** on a rest day, exercise day, and 24 hours after exercise. * - $p < 0.05$ and (*) - $p < 0.1$. **CMIE:** continuous moderate intensity exercise. **LV-HIIE:** low-volume high-intensity interval exercise. **SOD:** superoxide dismutase. **TBARS:** thiobarbituric acid reactive substances. Values are means ± SEM.

5.5 Discussion.

We report the novel finding that LV-HIIE performed 1 hour after consumption of a standard breakfast elicits a greater postprandial oxidative stress response compared to CMIE. Yet, over the 24-hour post-exercise period LV-HIIE improves glycaemic control to a similar extent as CMIE in overweight and obese adults. Furthermore, only LV-HIIE attenuated postprandial oxidative stress 24

hours after exercise. LV-HIIE consisted of significantly less work and time commitment compared to CMIE and therefore appears to be an effective exercise mode for incorporation into exercise programs designed to improve glycaemic control in overweight and obese populations including insulin resistant conditions like PCOS.

5.5.1 Acute exercise and 24-hour glycaemic control.

Previous research indicates that a single bout of endurance exercise improves insulin sensitivity in the hours after exercise (144, 247, 437), however the effects of HIIE on glycaemic control in sedentary overweight and obese populations are less clear. Laboratory based methods, such as the hyperinsulinaemic-euglycemic clamp and oral glucose tolerance test, may not reflect physiological insulin and glucose dynamics (295) and can underestimate functional improvements in glycaemic control (284). We report the novel finding that 24-hour post-exercise glycaemic control measured under free living conditions is improved with LV-HIIE to a similar extent as CMIE. The mechanisms for improved post-exercise glycaemic control with high-intensity exercise remain unclear, but may stem from increased muscle fibre recruitment (104), increased glycogen depletion and post-exercise glycogen re-synthesis (256), increased post-exercise oxygen consumption and energy expenditure (180, 387), and/or altered post-exercise redox homeostasis (362, 435).

Manders, et al. (260) previously reported that CMIE (60 minutes at 35% W_{max}) elicited greater improvements in 24-hour glycaemic control compared to higher-intensity exercise (30 minutes at 70% W_{max}) in participants with type 2 diabetes. The discrepancy in findings may be a result of the different populations investigated, participants with type 2 diabetes versus overweight/obese adults. However, Terada et al. (417) recently reported greater improvements in 24-hour glycaemic control and postprandial glycemia after fasted-state HIIE (15 x 1 minute at 100% VO_{2peak}; 3-minute active recovery periods) compared to work-matched CMIE treadmill exercise (60 minutes at 55% $VO2_{peak}$) in participants with type 2 diabetes. Furthermore, HIIE cycling (10 x 1 minutes at 90% W_{peak}; 1 min recovery periods at 15% W_{peak}) in overweight/obese adults elicits greater improvements in 24-hour glycaemic control compared to work-matched CMIE (30 minutes at 35% W_{peak}) (247). Combined with the current findings, HIIE is a

potent stimulus for improving glycaemic control, with potentially greater benefits occurring with HIIE of sufficient volume. In contrast, research exploring sprint-interval exercise reported no improvement in insulin sensitivity at 24 hours and 72 hours post-exercise (354, 466), although insulin sensitivity was improved 24 hours after an extended sprint work-matched to sprint-interval exercise (466). However, these studies did not measure glycaemic control during the post-exercise recovery period (354, 466). We extend previous findings by reporting that LV-HIIE, which consisted of substantially less time commitment and total work than CMIE, elicits similar improvements in 24-hour post-exercise glycaemic control.

Regulation of glycaemic fluctuations and postprandial glycaemia are important for the long-term maintenance of insulin sensitivity and lowered risk of metabolic disease (473). Similar to previous reports (247), we did not detect significant changes in glycaemic variability as measured by MAGE, SD, and CV, with either LV-HIIE or CMIE. It is possible that differences were not detected, as CGM readings have been reported to underestimate measures of glycaemic variability (5). Furthermore, greater glycaemic variability is strongly associated with a history of diabetes, suggesting that glycaemic variability may play less of a role in apparently healthy populations who have regular glycaemic control (376). Nevertheless, the significant reductions in hyperglycaemia, improved postprandial dinner response, and improved 24-hour average blood glucose and AUC, identify LV-HIIE as a beneficial exercise mode for improving overall glycaemic control in sedentary, overweight and obese individuals. Indeed, training programs incorporating LV-HIIE are reported to promote long term improvements in glycaemic control (141). We extend these findings by reporting that a single session of LV-HIIE or CMIE can have similar improvements in glycaemic control for up to 24 hours after exercise has ceased.

5.5.2 Redox status response to exercise performed 1 hour after breakfast.

Excessive oxidative stress leads to the development and progression of numerous pathologies including insulin resistance and type 2 diabetes (122, 436). Increased energy substrate availability following the consumption of a meal, results in elevated ROS production through mitochondrial membrane

electron leak and the formation of AGEs (122, 433). Excess ROS activate stress and mitogen activated protein kinase signalling pathways in insulin sensitive tissues contributing to the development of insulin resistance and type 2 diabetes (421, 473). However, research has also highlighted exercise-induced ROS as a prominent moderator of glycaemic control (358, 429).

We report increased postprandial oxidative stress (TBARS and hydrogen peroxide) and antioxidant activity (catalase and SOD activity) 1 hour after breakfast. In addition, hydrogen peroxide, catalase and SOD activity remain elevated 3 hours after breakfast. Interestingly, only CMIE attenuated this postprandial oxidative stress response as evident by decreased TBARS and hydrogen peroxide 1.5 hours after exercise compared to pre-exercise values, and decreased plasma TBARS 1.5 hours after exercise compared to the rest day. Taken together with previous work (59), our findings suggest that postprandial oxidative stress is attenuated by CMIE, possibly due to improved clearance of plasma glucose. Certainly, others have reported that CMIE (1 hour at 60% HR_{max}) performed 2 hours after a high fat meal attenuates postprandial oxidative stress in trained males (272). In contrast, others have reported that cycling at 65-70% heart rate reserve for 45-60 minutes did not attenuate postprandial oxidative stress (59, 277). The discrepancy in findings are likely related to the timing of meal ingestion, with improvements in postprandial oxidative stress occurring when exercise is performed in the hours after meal ingestion (272), whereas exercising prior to meal ingestion may be less effective (59, 277).

A novel finding was that plasma hydrogen peroxide and TBARS were elevated 1.5 hours after LV-HIIE (approximately 3 hours after breakfast) compared to the rest day. The mechanisms for the divergent oxidative stress response between LV-HIIE and CMIE are unclear. Oxidative stress is reported to be greater after higher-intensity exercise (120) and likely occurs through pathways independent of postprandial-induced oxidative stress (120, 122, 346). Thus, it is likely that elevated oxidative stress after LV-HIIE is a result of increased exercise-induced oxidative stress. Additionally, it is possible that comparatively lower blood glucose after CMIE may allow for decreased mitochondrial electron leak, AGE formation, and subsequent ROS production (122, 433, 473).

Exercise-induced oxidative stress and subsequent redox-sensitive protein signalling facilitate many of the health benefits of acute and regular exercise (324, 346, 358, 429). Furthermore, many of the metabolic health benefits of higher-intensity exercise occur during the delayed exercise recovery period (i.e. the day after exercise), potentially through alterations in redox status (136, 435). It is possible that increased exercise-induced oxidative stress after LV-HIIE may be beneficial. In support, only LV-HIIE attenuated postprandial oxidative stress 24 hours after exercise. Further research is required to elucidate the effect of LV-HIIE on redox status during the 24-hour post-exercise recovery period.

5.5.3 Glucoregulatory response to exercise performed 1 hour after breakfast.

Elevated postprandial glycemia is reported to play a role in the development of insulin resistance and metabolic disease (473). Similar to previous research (438), we demonstrate that a single session of CMIE performed 1 hour after breakfast attenuates postprandial glycemia. We extend previous findings by indicating that LV-HIIE also attenuates postprandial glycemia, despite consisting of considerably less total work and time-commitment. CMIE decreased whole blood glucose immediately after exercise compared to baseline and LV-HIIE. This reduction was transient as both LV-HIIE and CMIE elicited a similar decrease in whole blood and CGM glucose measures 1.5 hours after exercise. Circulating plasma catecholamine are reported to increase during high-intensity exercise which leads to a seven to eight-fold increase in hepatic glucose production compared to moderate intensity exercise (266). During recovery, catecholamine concentrations rapidly decrease, removing the catecholamine inhibition of glucose-stimulated insulin secretion (266). This leads to elevated plasma glucose and insulin levels after high-intensity exercise compared to moderate intensity exercise. This stress hormonal response may explain the comparatively higher glucose levels immediately after LV-HIIE, and higher post-exercise insulin levels throughout the recovery period, compared to CMIE. It is important to note that this elevated glucose response with LV-HIIE was transient and did not negatively impact the 24-hour improvements in glycaemic control post-exercise.

5.5.4 Limitations.

A strength of this study was the recruitment of inactive and overweight and obese men and women including a subgroup of women with PCOS. Although sex differences in glycaemic control (147) and postprandial oxidative stress have been reported (46), sample size and participant characteristics were well matched between LV-HIIE and CMIE exercise groups, and all females were tested in the early follicular phase of the menstrual cycle. A small sample size prevented subgroup analysis to explore whether particular groups (men, women and/or women with PCOS) had different glycaemic control responses to the exercise sessions. Despite this limitation, this study provides preliminary data to suggest that overweight and obese men, women, and women with PCOS, may be able to improve 24-hour glycaemic control with a single session of CMIE or LV-HIIE. Future research is warranted to investigate glycaemic responses in these subgroups. Plasma TBARS (malondialdehyde) and hydrogen peroxide have previously been used to reflect systemic postprandial oxidative stress (45, 46, 59). Nevertheless, future research would benefit from employing additional measures of oxidative stress in plasma such as the oxidized/reduced glutathione ratio or the direct measurement of ROS through spin trapping and electron spin resonance spectroscopy. Another potential study limitation is that the participants were not blinded to real-time CGM readings, which when prompted are displayed on an LCD screen. If possible, future studies should adopt a CGM system such as the iPro®2 Professional CGM (Medtronic MiniMed, Northridge, CA) which allows blinding of participants to real-time CGM readings. A potential limitation of the study is the natural variability of outcome measures between the two exercise groups which is inherent to a parallel design study. Despite appropriate matching of participant characteristics and the inclusion of the rest-control day for exercise day comparisons, future research would benefit from adopting a cross-over design study to confirm these findings. In summary, further cross-over randomized control trials are warranted to explore the acute and chronic impacts of LV-HIIE in larger groups of people in specific clinical populations, and using more diverse exercise protocols including that of work-matched CMIE.

5.5.5 Conclusion.

A single session of CMIE, but not LV-HIIE, attenuated postprandial plasma hydrogen peroxide and glycemia when performed 1 hour after breakfast consumption. Yet, LV-HIIE attenuated postprandial oxidative stress 24 hours after exercise and elicited similar improvements in 24-hour glycaemic control when compared to CMIE. Given its time-efficient nature, LV-HIIE may be an effective exercise mode to incorporate into exercise programs for the improvement of 24-hour glycaemic control in inactive, overweight and obese adults. Furthermore, measuring glycaemic control immediately after exercise may not accurately reflect functional improvements over the 24-hour post-exercise recovery period, especially with respect to high-intensity exercise. These findings also indicate that CGM is an effective and sensitive tool for measuring post-exercise improvements in glycaemic control under free-living conditions in normoglycemic populations. Future research should endeavour to adopt both CGM and laboratory based measures of glycaemic control.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS.

6.0 Thesis aims.

The overall aim of this thesis was to explore the effect of exercise-intensity on post-exercise redox status, skeletal muscle redox-sensitive protein signalling, and glycaemic control. Chapter 3 explored the effects of a single session of CMIE, HIIE, and SIE, on post-exercise plasma redox status, skeletal muscle redox-sensitive SAPK and insulin protein signalling. Chapter 4 explored whether HIIE induced changes in redox status and SAPK signalling would coincide with enhanced skeletal muscle insulin protein signalling and whole-body inulin sensitivity. The final experimental chapter (Chapter 5) compared the effects of a single session of LV-HIIE and CMIE on postprandial glycaemia and plasma redox status, and 24-hour glycaemic control under free-living conditions. The following section summarizes the main findings of each chapter, discusses the inherent limitations and practical implications of these findings, and provides recommendations for future research.

6.1 Summary of key findings.

In Chapter 3 we provided the novel finding that SIE, which consisted of substantially less total work than HIIE and CMIE, elicited greater plasma catalase activity and skeletal muscle NF-κB p65 phosphorylation in young recreationally active adults. Furthermore, despite consisting of considerably less total work, SIE elicited a similar increase in skeletal muscle JNK and p38 MAPK phosphorylation, and decrease in skeletal muscle PKC δ/θ phosphorylation, compared to CMIE and HIIE. Surprisingly, AS160^{Ser588} phosphorylation was markedly lower immediately after SIE, and was lower 3 hours after HIIE and CMIE.

In Chapter 4 we provided new evidence that insulin stimulation via the hyperinsulinaemic-euglycaemic clamp increases plasma antioxidant activity, decreases plasma oxidative stress, and increases 4-HNE, JNK, and p38 MAPK phosphorylation in skeletal muscle in middle-aged obese males. Importantly, insulin stimulated phosphorylation of JNK, NF-κB and p38 MAPK, occurred to a greater extent after HIIE and coincided with enhanced distal insulin signalling and whole-body insulin sensitivity.

In Chapter 5 we reported that a single session of LV-HIIE, consisting of substantially less total work and time commitment than traditional CMIE, improved 24-hour glycaemic control under free-living conditions to a similar extent in overweight and physically inactive adults. Both CMIE and LV-HIIE decreased postprandial glucose when performed 1 hour after breakfast, despite elevated oxidative stress after LV-HIIE. In contrast, LV-HIIE provided greater protection against postprandial oxidative stress ~24 hours after exercise.

6.2 Overall discussion.

Previous research has revealed a strong relationship between physical inactivity, obesity, oxidative stress, and the sustained activation of redox-sensitive JNK, p38 MAPK, NF-κB and PKC δ/θ pathways associated with the development and progression of cardiometabolic disease (436). Paradoxically, during and after exercise, similar protein signalling pathways are activated (120, 228), yet skeletal muscle adaptation and metabolic health are enhanced (346, 436).

Previous studies have reported HIIE to elicit similar, and in some cases greater exercise-mediated skeletal muscle adaptations compared to CMIE (141, 348, 460). The signalling pathways responsible for HIIE induced metabolic adaptation remain unclear, however may involve exercise-induced oxidative stress and increased skeletal muscle JNK, p38 MAPK and NF-κB signalling (228, 346). This thesis provides new evidence that a single session of SIE elicits greater post-exercise plasma catalase activity and NF-κB phosphorylation in skeletal muscle compared to HIIE and CMIE. Furthermore, SIE increased p38 MAPK and JNK phosphorylation, and decreased PKC δ/θ phosphorylation, to a similar extent as HIIE and CMIE despite consisting of considerably less total work. These findings demonstrate that increased p38 MAPK and JNK phosphorylation after CMIE were not further increased with higher-intensity intermittent exercise. It is possible that greater skeletal muscle adaptation often reported after HIIE and SIE training compared to CMIE may occur through alternative protein signalling pathways. In contrast, NF-κB phosphorylation and plasma catalase activity is responsive to intense exercise, highlighting a novel potential pathway for SIE mediated skeletal muscle adaptation. Furthermore,

although the role of exercise-induced AS160^{Ser588} in human glycaemic control remains elusive (62), this thesis provides evidence that skeletal muscle phosphorylation of AS160^{Ser588} in humans is decreased after acute exercise in an exercise-intensity and post-exercise time-course dependent manner.

This thesis explored the role of exercise-induced oxidative stress and skeletal muscle redox-sensitive SAPK signalling in human glycaemic control. Previous studies have reported ROS and SAPK signalling to play a role in glucose metabolism (34, 138, 253, 398, 420, 429). However, not all studies agree (182, 335) and only a few studies have investigated the effects of exercise-induced redox status and SAPK signalling on glycaemic control in humans (358, 429). This thesis provides new evidence that whole-body insulin stimulation in humans significantly increases skeletal muscle oxidative stress (4-HNE) and JNK, p38 MAPK and NF-κB phosphorylation.

It is possible that hyperinsulinemia during the insulin clamp may have resulted in negative feedback inhibition of the insulin signalling cascade and glucose uptake which is mediated in part through ROS and SAPK signalling (238, 421). In contrast, elevated SAPK signalling and ROS following insulin infusion may be a physiological response, as insulin stimulated increases in ROS and SAPK signalling are reported to be necessary for normal propagation of the insulin signalling pathway and insulin stimulated glucose uptake (34, 122, 257).

Similar to skeletal muscle, insulin stimulation in obese-middle aged males increased plasma antioxidant activity and decreased plasma oxidative stress. This redox shift elicited by insulin stimulation supports previous research reporting redox homeostasis as an important regulator of insulin stimulated glucose uptake (30, 111, 122, 219, 253, 257, 421). Interestingly, the redox shift elicited by acute exercise alone did not further contribute to the redox shift elicited by insulin stimulation. It is plausible that any potential additive effect may have been regulated by the endogenous antioxidant defence system during post-exercise insulin stimulation to maintain a redox environment conducive for insulin signalling and glucose uptake. Certainly, previous reports indicate that hydrogen peroxide can both attenuate and/or enhance insulin stimulated glucose uptake in-vitro depending on its concentration (195). Furthermore, altering redox homeostasis via exogenous oxidants and/or

antioxidants in cell culture, animals and/or humans during insulin stimulation have been reported to elicit a range of positive and/or negative responses with respect to insulin protein signalling and insulin sensitivity (34, 98, 156, 253, 327, 358, 421, 429). Taken together, our findings support the idea that redox homeostasis is tightly regulated in-vivo (349), particularly with respect to insulin stimulated glucose uptake (122).

A major finding of the thesis was that a prior bout of HIIE enhanced insulin stimulated phosphorylation of JNK, p38 MAPK and NF-κB, decreased phosphorylation of PKC δ/θ, and enhanced distal insulin protein signalling and whole-body insulin sensitivity. This thesis provides new evidence that supports increased JNK, p38 MAPK and NF-κB phosphorylation as a contributing mechanism for the post-exercise enhancement of insulin signalling and whole-body insulin sensitivity in obese middle-aged males.

In Chapter 4 we reported enhanced whole-body insulin sensitivity, assessed by hyperinsulinaemic-euglycaemic clamp, approximately 3 hours after a single session of HIIE. Although reliable and valid, research has indicated that laboratory based tests may not always reflect physiological (295) and functional changes (284) in glycaemic control which can persist for up to 24 hours after exercise has ceased (247). This thesis extends previous work by indicating that a single session of LV-HIIE, which consisted of substantially less total work and time commitment than CMIE, elicits similar improvements in 24-hour glycaemic control as measured by CGM in overweight and inactive adults. Furthermore, both CMIE and LV-HIIE decreased postprandial glucose when exercise was performed 1 hour after breakfast consumption, however only CMIE decreased postprandial oxidative stress. Importantly, elevated oxidative stress after LV-HIIE did not affect improvements in 24-hour glycaemic control. On the contrary, only LV-HIIE decreased postprandial oxidative stress 24 hours after exercise. These findings support previous reports that exercise-induced changes in systemic redox status may contribute towards the acute improvements (up to 24 hours) in glycaemic control (136, 362) and cardiovascular health (435).

As reported in this thesis, systemic oxidative stress transiently increases after both acute exercise and meal consumption. However, in contrast to the reported pathological effects of postprandial oxidative stress (473), exercise-

induced oxidative stress appears to be beneficial (346). The divergent effects may stem from the mechanism of ROS production. The pathological effects of oxidative stress are reported to primarily occur through mitochondrial dysfunction and excess mitochondrial ROS production (122). In contrast, the beneficial effects of exercise-induced ROS production are reported to primarily occur through alternative mechanisms such as NADPH oxidase and xanthine oxidase (197). Furthermore, ROS can have varying physiological effects depending on the biological context. For example, hydrogen peroxide exposure to hepatocytes leads to divergent effects on insulin protein signalling and glucose uptake depending on the concentration of hydrogen peroxide (195). In addition, manipulation of the exposure time of hydrogen peroxide to skeletal muscle cells leads to divergent effects on insulin protein signalling and glucose uptake, despite similar redox-sensitive protein signalling responses (34). Importantly, it was established that the subcellular localisation of redox-sensitive protein signalling was key to the divergent effects of hydrogen peroxide exposure time on glycaemic control (34). Taken together, in the context of the reported thesis findings, it is likely that the divergent response between acute exercise and overnutrition (meal consumption) occur through a combination of factors such as the source of ROS production, the exposure time of ROS, the specific type of ROS, and the subcellular localisation of ROS and redox-sensitive protein signalling.

Previous research has reported elevated systemic oxidative stress in populations at risk of cardiometabolic disease (446). Data provided within this thesis support a similar finding, with obese middle-aged males measuring significantly higher basal plasma oxidative stress and lower antioxidant activity compared to younger non-obese individuals (Appendix A: Figure 1). The role of chronic oxidative stress in the development and progression of disease has led to the use of antioxidant supplementation in an effort to mitigate oxidative stress and improve metabolic health, however empirical evidence is controversial (40, 41). Furthermore, therapeutic drug use which directly or indirectly attenuate oxidative stress, such as metformin, statins, resveratrol and antioxidant supplements, have in many cases been reported to interfere with and/or blunt certain exercise-induced redox-sensitive skeletal muscle and cardiometabolic health adaptations (158, 253, 258, 358). Certainly, attenuation of redox-

sensitive SAPK signalling with ROS inhibitors coincides with impaired skeletal muscle signalling pathways that are involved in mitochondrial biogenesis and endogenous antioxidant upregulation (154, 155, 181, 209, 281, 333). Our findings support previous studies, highlighting the important role of redox homeostasis and redox-sensitive SAPK signalling in metabolic health, and support the growing trepidation about the use of antioxidant supplementation with exercise (158).

In summary, this thesis advances our understanding of the role of redox biology in metabolic health and disease (346, 421). Chronic oxidative stress and sustained activation of SAPK signalling pathways leads to impaired glycaemic control and metabolic disease (34, 421). In contrast, data provided within this thesis supports a beneficial role for the exercise-induced transient activation of skeletal muscle redox-sensitive SAPK signalling and alterations in redox status for the improvement of metabolic health. Finally, this thesis provides evidence that supports SIE and HIIE as potent stimuli for exercise-induced SAPK signalling and enhancement of glycaemic control in both healthy populations and populations at risk of cardiometabolic disease.

6.3 Limitations and considerations.

This thesis provides new insight into the complex relationship between exercise, redox status, skeletal muscle redox-sensitive protein signalling, and metabolic health and disease in humans. However, interpretation of the data must be considered in the context of the limitations of the research.

6.3.1 General limitations.

Data presented within this thesis is limited by the recruitment of both male and female participants in Chapter 3 and 5, and male only participants in Chapter 4. Despite sex differences in basal redox homeostasis (43), which may occur independent of the menstrual cycle phase (75), exercise-induced oxidative stress responses are reported to be similar between sexes (151). The inclusion of females in Chapter 3 and 5 is therefore unlikely to have influenced the exercise-induced systemic redox status response reported. On the other hand, sex differences in glycaemic control and postprandial oxidative stress have been reported between males and females (43, 47, 146, 147, 279). The

measurement of glycaemic control and postprandial oxidative stress in Chapter 5 may therefore be limited by the combined analysis of both males and females. The inclusion of only middle-aged males in Chapter 4 also delimits the reported findings to that of the specific population investigated.

Previous studies have reported variations in training adaptations and skeletal muscle substrate metabolism between male and females (23, 140, 255). Although the acute post-exercise p38 MAPK skeletal muscle signalling response to sprint exercise is similar between males and females (131), the effects of sex on other protein kinases are unknown. To minimize the effect of hormonal fluctuations on outcome measures, females were tested in the early follicular phase of the menstrual cycle (2-7 days after the onset of menses). Nevertheless, further research is required to confirm the findings presented within this thesis in both males and females individually.

Exercise induced oxidative stress and SAPK signalling, including p38 MAPK, are reported to occur to a greater extent after exercise in untrained and obese individuals (380, 445, 447, 482). Likewise, exercise-induced oxidative stress and skeletal muscle protein signalling responses are attenuated in subsequent sessions of identical exercise (7, 52, 90, 311, 329). Thus, the acute exercise-induced oxidative stress and protein signalling response varies depending on the current training and health status of participants. Findings of Chapter 3, 4 and 5, are therefore limited to the specific populations studied, and limited to a single isolated session of exercise.

The acute activation of protein signalling pathways in skeletal muscle does not always reflect functional changes in protein synthesis and/or adaptations with chronic exercise training (79, 451). Likewise, the acute exercise-induced upregulation of numerous mitogenic pathways are reported to occur much later in the recovery phase (184, 329). As such, findings of Chapter 3, 4 and 5, are limited to the acute response of a single session of exercise, and may not capture all changes in the exercise recovery period. Further research is required to confirm whether the reported acute changes in redox status and SAPK signalling translate to long term skeletal muscle and metabolic health adaptations.

The indirect measurement of ROS in plasma via oxidative stress biomarkers is a common practice for determining systemic redox status in a biological sample. However, non-specificity (125, 199, 276) and post-exercise time-course differences (280) indicate that multiple biomarkers should be measured when determining tissue redox homeostasis (172, 201). Plasma TBARS, hydrogen peroxide, catalase and superoxide dismutase activity have been used extensively in previous research to explore plasma oxidative stress and antioxidant activity in humans (35, 45-49, 59, 66, 120, 121, 380). As such, the plasma redox status assays adopted provide an adequate surrogate of gross systemic oxidative stress and antioxidant activity. Nevertheless, future research would benefit from employing additional measures of oxidative stress in plasma such as the oxidized/reduced glutathione ratio, F2-isoprostanes, or the direct measurement of ROS through spin trapping and electron spin resonance spectroscopy (125).

6.3.2 Chapter 3 limitations.

A limitation of the experimental Chapter 3 was the absence of measurements for post-exercise skeletal muscle adaptations such as gene expression and/or protein synthesis. Furthermore, the effect of decreased AS160^{Ser588} phosphorylation after exercise on glycaemic control was not measured. In Chapter 3 exercise-intensity did not significantly influence post-exercise plasma oxidative stress, a finding in contradiction to previous reports (161, 162, 237, 254). We speculate that the intermittent nature of HIIE and SIE may have allowed for sufficient time for endogenous antioxidant defences to mitigate exercise-induced ROS and oxidative stress. However, a small sample size (n=8) may have limited interpretation of findings. Certainly, mean values for post-exercise hydrogen peroxide after HIIE and SIE, and TBARS immediately after SIE, were higher compared to CMIE. Further research is required to confirm these findings using a larger cohort of participants, both males and females.

To minimise the effect of insulin and glucose loading (i.e. breakfast consumption) on outcome measures, participants performed the exercise bouts in a fasted state. Previous studies have shown that fasting prior to exercise can lead to differential effects on exercise-induced improvements in postprandial

oxidative stress and glycemia (59, 272, 277, 417). As such, future research is required to confirm the current findings after non-fasted exercise.

6.3.3 Chapter 4 limitations.

One of the limitations of Chapter 4 was the absence of an exercise-only control trial. This was primarily due to the invasiveness and resource intensive nature of the study. It is therefore difficult to interpret whether increased phosphorylation of stress protein kinase signalling after exercise and insulin stimulation was an additive effect, or due to prior exercise enhancing insulin stimulated phosphorylation of these proteins. In support of the latter, p38 MAPK and NF-kB phosphorylation were significantly increased 3 hours after exercise (and insulin stimulation) in Chapter 4, whereas at the same time point in Chapter 3, which involved exercise-only, phosphorylation was not significantly elevated compared to baseline. Others have also reported that p38 MAPK phosphorylation does not remain elevated 3 hours after HIIE in the absence of insulin stimulation (29, 81, 142, 248).

The hyperinsulinaemic-euglycaemic clamp is considered to be the gold standard for measuring insulin sensitivity (96). However, hyperinsulinemia induced during insulin infusion may not reflect physiological insulin and glucose dynamics (295). Furthermore, hyperinsulinemia can activate stress protein kinase signalling through negative feedback inhibition of the insulin signalling cascade (164, 238, 365, 459, 480). Certain phosphorylation sites of AS160, such as Ser588, are activated by a hyperinsulinemic-euglycaemic clamp but not in the presence of physiological levels of insulin elicited by ingestion of a meal (427). Further research is required to confirm the outcomes of Chapter 4 after the ingestion of a standard meal. Sample size may be considered a limiting factor of Chapter 4. Previous invasive studies have however reported similar changes in whole-body insulin sensitivity and skeletal muscle SAPK signalling with similar or fewer participants (420, 429). Only a single marker of oxidative stress in skeletal muscle was measured in Chapter 4. Multiple biomarkers of oxidative stress in skeletal muscle would allow for greater interpretation of the effect of exercise and insulin stimulation on skeletal muscle redox status.

A limitation of Chapter 4 was the observational and associative nature of the study. To provide greater exploration of the role of redox-sensitive protein

signalling and insulin sensitivity, inhibition of ROS and/or SAPK signalling by administration of reducing compounds and/or anti-inflammatory drugs in humans, animals and/or cell culture would allow for greater mechanistic insight (271).

Another limitation of Chapter 4 was that the hyperinsulinaemic-euglycaemic clamp on the rest day was not conducted at the same time of day as the exercise trial (approximately 1.5 hours earlier in the morning). It is possible that this minor discrepancy in circadian timing and metabolic fasting between the two sessions may have confounded the results. Additionally, the order of session 1 (the rest day) and session 2 (the exercise day) were not randomized. The primary reason for this was due to safety concerns by the ethics committee to establish a baseline of insulin sensitivity prior to measuring the effects of high-intensity interval exercise in obese middle-aged males. However, post-exercise whole-body insulin sensitivity was enhanced by approximately ~34-40% and stress protein kinase signalling >100%, as such it is unlikely that differences in circadian timing and/or the lack of randomisation would account for these affects. Nevertheless, future research should endeavour to match circadian timing between exercise sessions and randomise interventions where practical.

6.3.4 Chapter 5 limitations.

In Chapter 5, the rest and exercise days were not randomized and a parallel groups design was implemented. Ideally a cross-over design would be employed and the rest and exercise sessions randomized, separated by a minimum of 1 week for males and approximately 28 days for females. This was not possible in Chapter 5 as the CGM sensors were rated to record for up to a maximum of 7 days (only 4 days in our hands; data not shown). Randomisation of trial day would require the insertion of multiple, separate CGM sensors introducing intra-measurement error. The use of a parallel design and assigning the rest day prior to the exercise day ensured that only one CGM sensor was required, decreased the total time commitment of participants, and made sure that measurements on the rest day were not influenced by the exercise session.

Participant's diet and exercise diaries over the consecutive 4-day experimental phase did not indicate additional consumption of food or exercise other than

that prescribed. It is however possible that participants did not record additional physical activity and/or food consumed, posing a potential limitation of the study. Nevertheless, the measurement of glycaemic control under free living conditions provides a functional and practical assessment of glycaemic control in humans and is considered a strength of Chapter 5. In the future, recording of physical activity via activity monitors in conjunction with exercise and food diaries may prove beneficial.

In Chapter 5, the CMIE group consisted of 38 minutes of continuous cycling exercise. This chosen exercise duration was the direct result of the initial study design which included an additional HIIE cycling group (4 x 4 min at 80% Wmax, with 2 min recovery periods). During the initial stages of data collection, the CMIE group was work-matched to the HIIE group which equated to 38 minutes of CMIE. However, due to participant recruitment and limited resources, the HIIE group was dropped early on in the study in favour of focusing on only the LV-HIIE and CMIE exercise groups. However, as data had already been collected from multiple participants, it was necessary to maintain the 38 minutes for all subsequent participants.

Due to the parallel design of Chapter 5, it was necessary to match the CMIE and LV-HIIE groups based on BMI and sex. Participants were recruited over a number of months/years, and involved females for which their testing availability was largely dependent on timing of the menstrual cycle. In addition, the resource intensive nature of the data collection procedures (e.g. four consecutive days of testing with CGM, blood samples, diet control etc.) ensured that it was not possible to recruit and test all the participants at once. This prevented the common randomisation procedure of block mode randomisation, in which participants are first recruited, and then randomised in blocks prior to commencement of the study or throughout stages of the study. As such, a minimisation randomisation model was adopted (424). Participants were initially randomised to LV-HIIE or CMIE by chance. This randomisation continued while monitoring the group averages of BMI and allocation frequency of male and females. In the event that one group received a participant with a BMI or frequency of Sex considerably above or below the group average/norm, another participant of similar characteristics would be consciously placed in the

other group to ensure group equilibrium. Participants were therefore sometimes allocated their group (HIIE or CMIE) not purely by chance, but by determining in which group inclusion of the participant would minimise any differences in the confounding factors of BMI and sex. The minimisation randomisation model is deemed an acceptable and often preferred method for randomisation in clinical trials (424). Nevertheless, this method may include some allocation bias, as allocation is conducted during the study. However, considering the design of the study, a minimisation randomisation model appears to be the most appropriate method for randomisation.

6.4 Practical implications.

Society faces a global epidemic of increased physical inactivity and obesity, conditions which carry substantial health and financial burden at an individual, national, and international level. Findings presented within this thesis support the ubiquitous role of physical activity for the enhancement of metabolic health, indicating that even a single session of exercise can elicit favourable improvements in glycaemic control. Oxidative stress and redox-sensitive SAPK signalling were once thought to be exclusively deleterious. This thesis adds new evidence that supports skeletal muscle SAPK signalling as a beneficial moderator of glycaemic control. These findings are important, and may in-part explain the reported negative effects of antioxidant supplementation on exercise adaptation and metabolic health.

In addition, this thesis highlights that SIE and LV-HIIE, exercise modes which consist of substantially less time and total work than traditional recommendations of CMIE, are potent stimuli for exercise-induced activation of SAPK signalling and enhancement of glycaemic control. Considering that “lack of time” is a commonly perceived barrier to exercise (430), lower-volume, higher-intensity, and shorter duration exercise, may be an attractive and exercise model to incorporate into exercise programs for time-restricted populations. In summary, this thesis supports the existence of a redox paradox, extending previous knowledge by highlighting the importance of exercise-induced oxidative stress and redox-sensitive SAPK signalling in human metabolic health.

6.5 Future directions.

This thesis advances our current understanding of acute exercise and mechanisms contributing to enhanced glycaemic control. However, certain limitations exist and future research is required to further explore the role of redox biology in health and disease. Future research would benefit by employing a combination of human primary cell culture and animal research to investigate both the mechanisms and physiological relevance of the reported thesis findings in humans (61, 155). Future research would benefit by complimenting laboratory based techniques for measuring insulin sensitivity with functional measures, such as the continuous glucose monitor, to ascertain the practical relevance of findings (284, 295).

Reducing compounds, including antioxidant supplements, have been relatively effective for investigating the role of ROS in glycaemic control (358), however in some cases the effects are only marginal (429). The biological effect of antioxidant supplementation in humans is confounded by the type of antioxidant compound/s used, the dose used, the timing of supplementation, and the often non-specific and/or ineffective action of antioxidant supplementation for ROS inhibition in humans (170, 262, 270, 271, 302). Specific antioxidants, such as SS31 and MitoQ which target mitochondrial ROS production, may prove more useful to isolate the role or redox biology in health and disease (361). However, the use of these compounds in humans are at best contradictory (395) or yet to be approved for use in human clinical trials. Future research is required to elucidate potential reducing compounds that are safe, and effective in humans, prior to their use as a mechanistic agent for the exploration of human redox biology in health and disease.

Data presented within this thesis supports the previously reported dual role of redox signalling in human pathology and physiology (30, 253, 421). Certainly, it appears that redox signalling occurs on a spatial-temporal paradigm that involves not only the concentration of ROS (195), but also the type of ROS, the organs and organelles involved, and the spatial-temporal distribution of ROS (30, 34). Berdichevsky, et al. (34) reported that chronic and acute oxidative stress elicit opposite effects on insulin protein signalling and glucose uptake in cell culture. Immunohistochemistry revealed that the differential effect was likely

mediated through subcellular redistribution of phosphorylated JNK from the cytoplasm into the nucleus (34). Future research would benefit from immunohistochemistry analysis of human tissue to explore the redox paradox between transient oxidative stress (exercise-induced), chronic oxidative stress (physical inactivity/excess nutrition intake), and the role of SAPK signalling in human health and disease.

The findings presented within this thesis are limited to the acute investigation of a single session of exercise. The benefits of acute and chronic exercise training on redox status and metabolic health may occur through independent, but interrelated SAPK signalling pathways (155, 253, 358, 362). Future research investigating HIIE would benefit from exploration of both the acute and the long-term training exercise responses of redox status, SAPK signalling, and metabolic health.

Early studies, and the majority of current findings, rely primarily on associations and the assumption that increased/decreased ROS and/or markers of oxidative stress are reflective of, or are likely to lead to, increased/decreased redox signaling (261). Certainly, studies inhibiting or increasing ROS have been useful for establishing a relationship between ROS and certain biological outcomes such as glycemic control and exercise adaption (181, 209, 358, 429). However, in the absence of specific redox signaling measurements such as protein cysteine oxidation or S-nitrosylation (267, 283), research studies are limited in their capacity to elucidate specific redox cellular signaling networks which are complex, compartmentalized, and spatiotemporally regulated (262). Future studies utilizing modern redox proteomics are required to establish the reversible, and in some cases irreversible, redox regulation of kinases, phosphatases, transcription factors and co-activators, thus establishing the “true” redox signaling role of exercise-induced ROS (163, 262, 263, 298, 381, 467). Furthermore, not all ROS are equal in their capacity to exert signaling effects (185). Future studies investigating exercise-induced oxidative stress should therefore strive to identify the specific ROS involved, which can be achieved through the use of robust techniques such as electron spin resonance, targeted fluorescent probes, and mass spectrometry (20, 78, 99, 196, 477).

Despite their non-specificity and/or inability to adequately reflect redox signaling, the measurement of ROS, oxidative stress and/or antioxidant activity in a biological sample provides insight into the effects of an intervention (e.g. exercise) on redox homeostasis, and remains a useful biomarker of overall health and disease (261). As such, a combination of both traditional measures of redox biomarkers, the direct measurement of ROS, redox-sensitive protein signaling, and specific redox proteomics, will likely provide a robust investigation of exercise-induced ROS and subsequent redox signaling.

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APPENDICES

Appendix A: Fasting plasma redox status of participants.

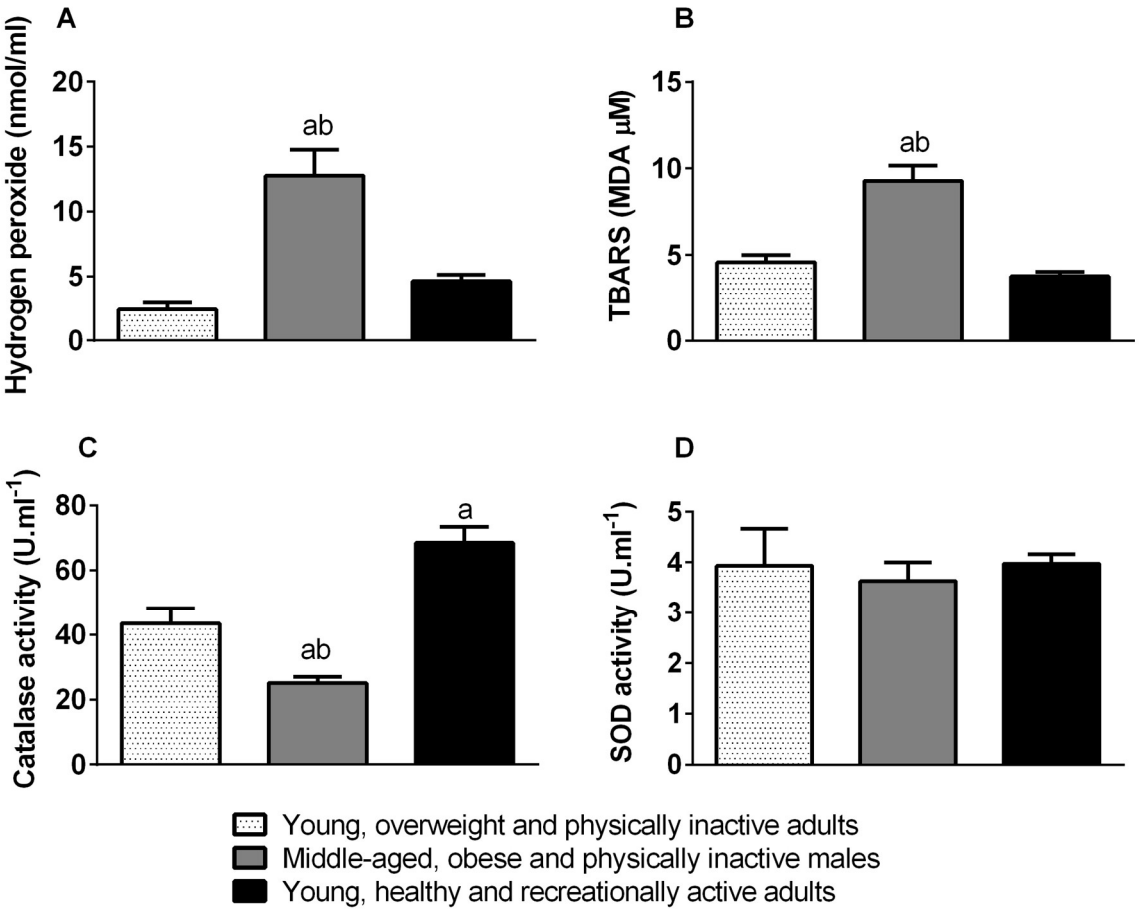

Figure 6.1: Fasting plasma oxidative stress and antioxidant activity of healthy populations and populations at risk of cardiometabolic disease. Fasting plasma hydrogen peroxide **(A)**, TBARS **(B)**, catalase activity **(C)**, and SOD activity **(D)**. a – $p < 0.05$ compared to young, overweight and physically inactive adults; b – $p < 0.05$ compared to young, healthy recreationally active adults.

Appendix B: Publications.

PLOS ONE

RESEARCH ARTICLE

The effect of exercise-intensity on skeletal muscle stress kinase and insulin protein signaling

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Data Availability Statement: Data are available from the Victoria University Institutional Online Database for researchers approved by the Victoria University Human Research Ethics Committee who meet the criteria for access to confidential data. Raw de-identified data can be accessed upon request to the corresponding author pending approval by the VUHREC.

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Abstract

Background

Stress and mitogen activated protein kinase (SAPK) signaling play an important role in glucose homeostasis and the physiological adaptation to exercise. However, the effects of acute high-intensity interval exercise (HIIE) and sprint interval exercise (SIE) on activation of these signaling pathways are unclear.

Methods

Eight young and recreationally active adults performed a single cycling session of HIIE (5 x 4 minutes at 75% W_{max}), SIE (4 x 30 second Wingate sprints), and continuous moderate-intensity exercise work-matched to HIIE (CMIE; 30 minutes at 50% of W_{max}), separated by a minimum of 1 week. Skeletal muscle SAPK and insulin protein signaling were measured immediately, and 3 hours after exercise.

Results

SIE elicited greater skeletal muscle NF-κB p65 phosphorylation immediately after exercise (SIE: ~40%; HIIE: ~4%; CMIE; ~13%; $p < 0.05$) compared to HIIE and CMIE. $AS160^{Ser588}$ phosphorylation decreased immediately after HIIE (~-27%; $p < 0.05$), and decreased to the greatest extent immediately after SIE (~-60%; $p < 0.05$). Skeletal muscle JNK (~42%; $p < 0.05$) and p38 MAPK (~171%; $p < 0.05$) phosphorylation increased, and skeletal muscle Akt^{Ser473} phosphorylation (~-32%; $p < 0.05$) decreased, to a similar extent immediately after all exercise protocols. $AS160^{Ser588}$ phosphorylation was similar to baseline three hours after SIE (~-12%; $p > 0.05$), remained lower 3 hours after HIIE (~-34%; $p < 0.05$), and decreased 3 hours after CMIE (~-33%; $p < 0.05$).

Conclusion

Despite consisting of less total work than CMIE and HIIE, SIE proved to be an effective stimulus for the activation of stress protein kinase signaling pathways linked to exercise-mediated

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adaptation of skeletal muscle. Furthermore, post-exercise AS160^{Ser588} phosphorylation decreased in an exercise-intensity and post-exercise time-course dependent manner.

Introduction

High-intensity interval-exercise (HIIE) and sprint-interval exercise (SIE) are reported to elicit comparable, and in some cases, greater improvements in measures of glycemic control, oxidative stress, and mitochondrial biogenesis, compared to continuous moderate-intensity exercise (CMIE) [1–5]. The mechanisms for improved skeletal muscle adaptation after HIIE and SIE are unclear, but may involve exercise-induced stress protein kinase signaling [6–9].

Physical inactivity and excess adipose tissue can lead to the sustained activation of mitogen and stress-activated protein kinases (SAPK), in-part through increased mitochondrial electron leak and the subsequent production of reactive oxygen species (ROS) [10, 11]. Important ROS sensitive SAPK proteins include c-Jun N-terminal kinases (JNK), p38 mitogen-activated protein kinases (p38 MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Sustained activation of these protein signaling pathways leads to impaired insulin sensitivity in part through serine phosphorylation of the insulin receptor substrate 1 (IRS-1), IRS-1 degradation and attenuation of distal insulin signaling proteins such as Akt substrate 160 (AS160) [11–13]. Paradoxically, acute exercise also results in increased ROS production [14], albeit transiently and predominantly through NADPH oxidase superoxide anion production [15], which is reported to contribute to the transient activation of SAPK signaling in skeletal muscle [16]. In contrast to the sustained activation of SAPK signaling, the transient activation following acute exercise coincides with greater AS160 phosphorylation post-exercise and improved insulin sensitivity [17–19]. Furthermore, exercise-induced SAPK signaling is also linked to the activation of skeletal muscle transcription factors and coactivators that lead to skeletal muscle adaptation and long-term improvements in cardiometabolic health [2, 5, 16, 20].

Although HIIE and SIE training are reported to elicit equivalent and in some cases superior exercise-mediated cardiometabolic adaptations when compared to CMIE [1, 2, 5], the effects of acute HIIE and SIE on post-exercise skeletal muscle SAPK signaling are equivocal. For example, greater metabolic fluctuations induced through intermittent exercise are considered to elicit greater post-exercise p38 MAPK phosphorylation [21]. However, previous studies have reported similar exercise-induced p38 MAPK phosphorylation after acute work-matched HIIE, SIE, and continuous exercise [22, 23]. The effects of low-volume SIE, compared to higher-volume HIIE work-matched to continuous exercise of moderate-intensity, on post-exercise skeletal muscle p38 MAPK phosphorylation are unknown. Furthermore, skeletal muscle JNK and NF-κB phosphorylation and post-exercise insulin protein signaling have yet to be explored after acute HIIE and SIE.

We compared the effects of a single session of HIIE, SIE, and CMIE work-matched to the HIIE, on skeletal muscle SAPK and insulin protein signaling. It was hypothesized that SIE and HIIE would elicit greater skeletal muscle SAPK and distal insulin protein signaling.

Materials and methods

Participants

Eight recreationally active adults, 6 males and 2 females, volunteered to participate in this randomized cross-over study. Participant characteristics are reported in Table 1. Exclusion

Table 1. Descriptive characteristics of participants.

Variable	N = 8
Participants	6 males and 2 females
Age (years)	25 ± 2
Height (cm)	179.3 ± 2.9
Weight	79.4 ± 2.1
BMI ($kg \cdot m^{-2}$)	25 ± 1
W_{max} during GXT (W)	327 ± 25
Max heart rate during GXT (BPM)	183 ± 4
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	48.4 ± 4.0

Values are mean ± SEM.

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criteria for participation included smoking, musculoskeletal or other conditions that prevent daily activity, symptomatic or uncontrolled metabolic or cardiovascular disease, and females taking oral contraception. To minimize the effect of hormonal fluctuations on outcome measures, females were tested in the early follicular phase of the menstrual cycle (2–7 days after the onset of menses). Verbal and written explanations about the study were provided prior to obtaining written informed consent. This study was approved by the Victoria University Human Research Ethics Committee and carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans [24].

Participants were asked to abstain from physical activity (~72 hours), alcohol and caffeine consumption (~24 hours) prior to each trial. Twenty-four hours before their first trial volunteers were asked to consume their habitual diet which was recorded in a diet diary and replicated in their subsequent trials. Participants completed a screening session prior to completing the three different exercise protocols in a randomized crossover fashion, separated by a minimum of 1 week for males and ~4 weeks for females (Fig 1).

Screening and preliminary testing

Participants were screened via a medical history and risk assessment questionnaire. Eligible participants underwent anthropometric measurement (height and weight) and completed a graded exercise test (GXT) on a cycle ergometer (Velotron, USA) to measure peak aerobic capacity (VO_{2peak}) and maximal power output (W_{max}). The GXT protocol consisted of 1-minute cycling stages at 50 watts which increased by 25 watts every minute until participants were unable to maintain a cycling cadence of 60 RPM or greater. Expired gases were collected and analyzed via an indirect calorimetry system (Moxus Modular VO_2 System, USA). The W_{max} obtained during the GXT was used to calculate the workload for the three exercise protocols.

Experimental phase

On three separate occasions participants reported to the laboratory in the morning after an overnight fast. A resting muscle biopsy and venous blood sample were taken prior to participants undergoing their randomized exercise protocol (SIE, HIIE or CMIE). Immediately following the acute session of exercise, a muscle biopsy and venous blood sample were taken, and participants rested on a bed for three hours. A third muscle biopsy was taken 3 hours after exercise and venous blood samples were taken in the middle of the exercise session, immediately after exercise, and 10 minutes, 30 minutes, 1 hour, 2 hours and 3 hours after exercise.

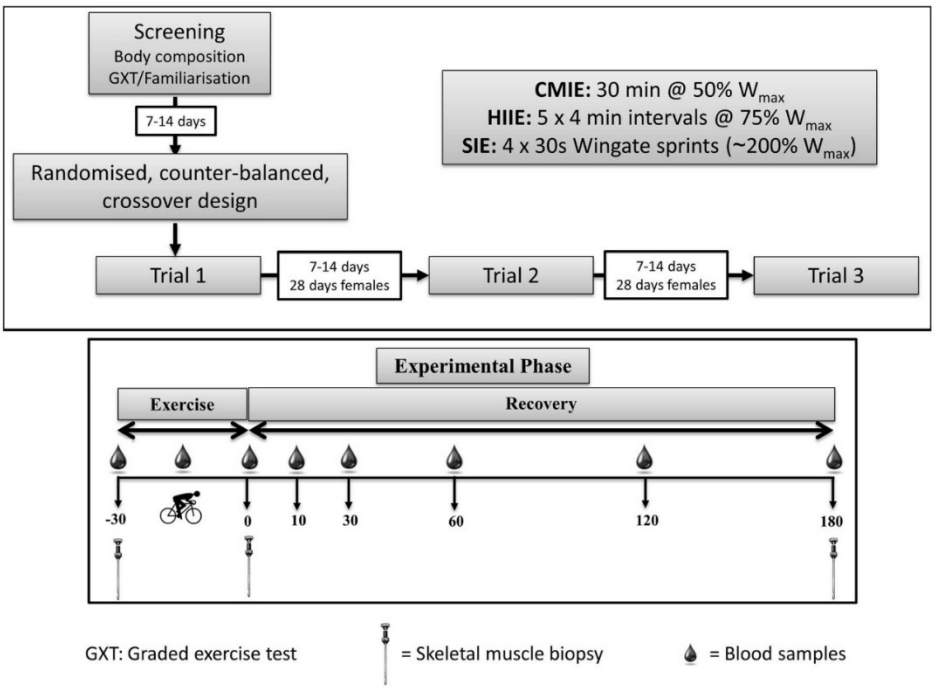

Fig 1. Schematic overview of research methodology. After initial screening and determination of W_{max} and VO_{2peak}, participants underwent three exercise sessions, separated by 7–14 days (~28 days for females), in a randomized crossover fashion. Venous blood and skeletal muscle samples were taken at time-points indicated in the figure. CMIE: continuous moderate-intensity exercise. HIIE: high-intensity interval exercise. SIE: sprint interval exercise. GXT: graded exercise test.

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Exercise protocols

All exercise sessions were performed on a Velotron cycle ergometer. The SIE protocol consisted of 4 x 30 second all-out (Wingate) cycling sprints, interspersed with 4.5-minute passive recovery periods. Pedaling resistance for the SIE was determined as a torque factor relative to body mass which was optimized during the familiarization session. The HIIE protocol consisted of 5 x 4-minute cycling bouts at 75% of W_{max} (~77% of VO_{2peak}), interspersed with 1-minute passive recovery periods. The CMIE protocol consisted of continuous cycling for 30 minutes at 50% of W_{max} (~54% of VO_{2peak}), equating to the same total work performed (294 ± 23 kJ) in the HIIE protocol.

Skeletal muscle and blood sampling

Muscle samples were obtained from the vastus lateralis under local anesthesia (Xylocaine 1%, Astra Zeneca, Australia) utilizing a Bergström needle with suction [25]. The samples were immediately frozen in liquid nitrogen and stored at -80˚C until analysis. Venous blood was collected from an antecubital vein via an intravenous cannula and analyzed immediately for blood glucose and lactate using an automated analysis system (YSI 2300 STAT Plus™ Glucose & Lactate Analyzer).

Skeletal muscle protein analysis

To avoid the potential loss of total cellular protein that can occur with centrifugation [26, 27], phosphorylation and abundance of specific proteins in whole muscle lysate were determined with all constituents present (i.e. no centrifugation). Whole muscle lysate was analyzed as previously reported [19]. In brief, thirty cryosections of skeletal muscle (20 μm) were homogenized in buffer (0.125M TRIS-HCL [pH 6.8], 4% SDS, 10% Glycerol, 10mM EGTA, 0.1M DTT, and with 0.1% v/v protease and phosphatase inhibitor cocktail [#P8340 and #P5726, Sigma Aldrich]). Total protein content of muscle lysate was determined using the commercially available Red 660 Protein Assay kit with SDS neutralizer as per the manufacturer's instructions (Red 660, G-Biosciences, St. Louis, MO, USA). Eight μg of protein was prepared in 3 μl of Bromophenol blue (1%), heated for 5 minutes at 95˚C and separated by 7.5% Criterion™ TGX™ Pre-Cast Gels. The separated proteins were transferred to a polyvinylidene difluoride membrane and blocked with Tris-Buffered Saline-Tween (TBST) and 5% skim milk for 1 hour. Membranes were washed (4 x 5 minutes) with TBST and incubated at 4˚C overnight with the following primary antibodies: phospho-SAPK/JNK (Thr183/Tyr185; CST #9251), SAPK/JNK (CST #9252), phospho-p38 MAPK (Thr180/Tyr182; CST #9211), p38 MAPK (CST #9212), phospho-NF-κB p65 (Ser536; CST #3033), NF-κB p65 (CST #8242), IκBα (CST #4814), phospho-IRS-1 (Ser307 in human; CST #2384), phospho-AS160 (Ser588; CST #8730), AS160 (CST #2447), phospho-Akt (Ser473; CST #9271), Akt (#9272), and IRS-1 (Millipore, 06–248). After incubation, membranes were washed with TBST and incubated for 1 hour at room temperature with appropriate dilutions of horseradish peroxidase conjugated secondary antibody. Membranes were re-washed and incubated in SuperSignal West Femto Maximum Sensitivity substrate for 5 minutes prior to imaging. After imaging, membranes were stained via a modified Coomassie staining protocol [19]. All densitometry values are expressed relative to a pooled internal standard and normalized to the total protein content of each lane obtained from the modified Coomassie staining protocol. Where appropriate, phosphorylated proteins are expressed relative to specific total protein content.

Statistical analysis

Data were checked for normality and analyzed using Predictive Analytics Software (PASW v20, SPSS Inc., Chicago, WI, USA). Comparisons of multiple means were examined using a repeated measures analysis of variance (exercise protocol x time point). Post hoc analysis of significant interaction and main effects were performed using Fisher's protected LSD test. All data are reported as mean ± standard error of mean (SEM) and statistical analysis conducted at the 95% level of significance ($p \leq 0.05$). Trends were reported when p-values were greater than 0.05 and less than 0.1.

Results

Blood glucose and lactate

Significant interaction effects ($p<0.05$) were detected for blood glucose and lactate ($p<0.05$). Post-hoc analysis revealed that compared to baseline, blood glucose was significantly elevated ($p<0.05$) after HIIE, and to the greatest extent after SIE (Fig 2). Furthermore, post-hoc analysis revealed that compared to baseline, blood lactate was elevated after CMIE, HIIE, and to the greatest extent after SIE (Fig 2).

Skeletal muscle SAPK signaling

A significant interaction effect ($p < 0.05$) was detected for NF-κB p65 phosphorylation. Post-hoc analysis revealed significantly greater ($p < 0.05$) NF-κB p65 phosphorylation immediately

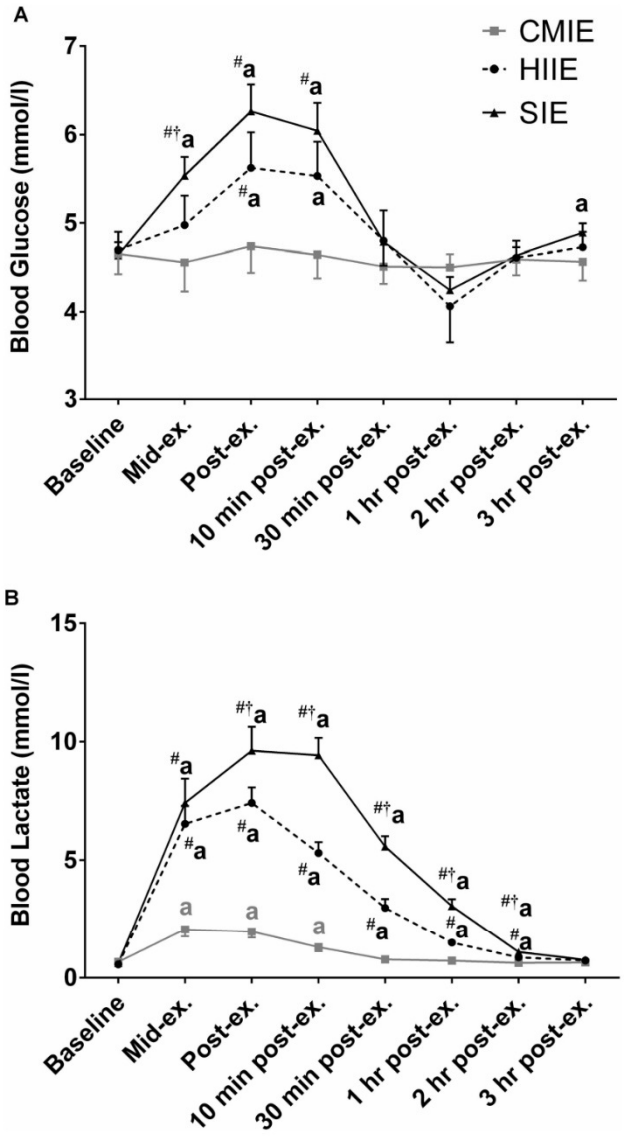

Fig 2. Blood lactate and blood glucose during and after exercise. (A) Blood lactate and **(B)** blood glucose response to high-intensity interval exercise **(HIIE)**, sprint-interval exercise (SIE), and continuous moderate-intensity exercise **(CMIE)**. a = $p < 0.05$ compared to baseline. Significantly different ($p < 0.05$) at equivalent time point vs # = CMIE and † = HIIE

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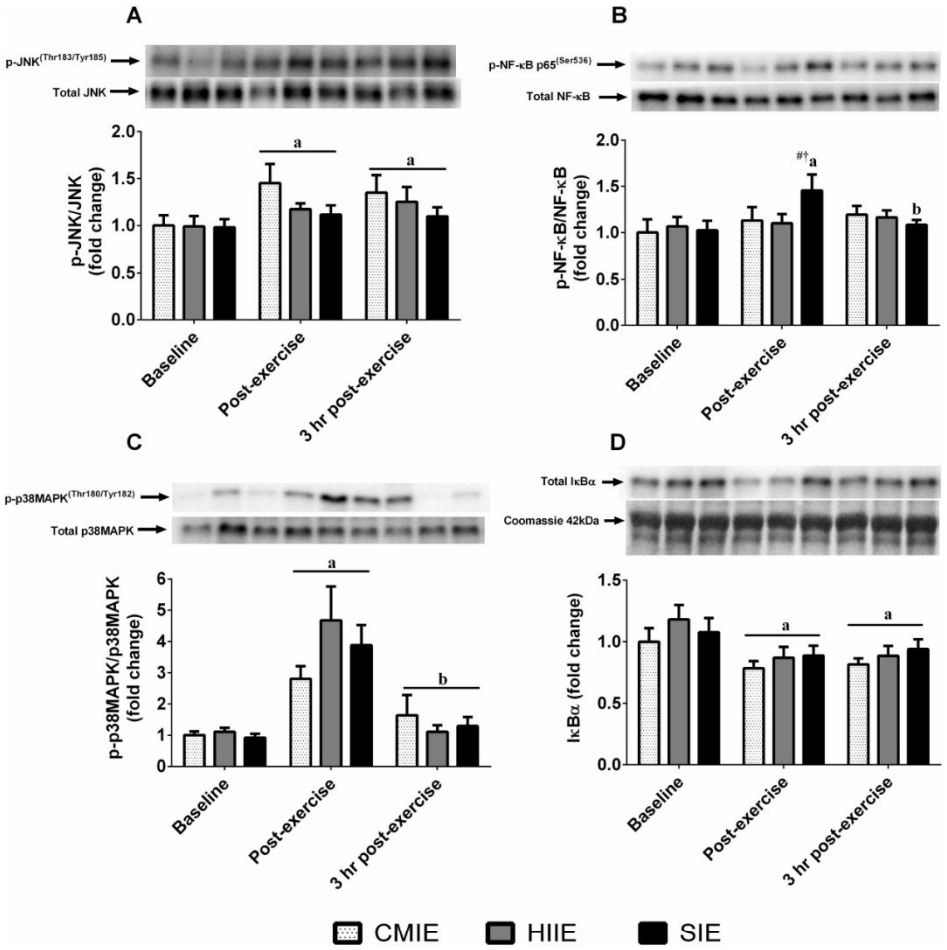

Fig 3. Skeletal muscle SAPK signaling. Skeletal muscle protein phosphorylation relative to total protein content of **(A)** JNK$^{Thr183/Tyr185}$, **(B)** NF-κB p65^{Ser536}, **(C)** p38 MAPK$^{Thr180/Tyr182}$, and total protein content of **(D)** IκBα relative to Coomassie protein content, after high-intensity interval exercise **(HIIE)**, sprint interval exercise **(SIE)**, and continuous moderate-intensity exercise **(CMIE)**. a = p < 0.05 compared to baseline; b = p < 0.05 compared to post-exercise. Significantly different (p < 0.05) at equivalent time point vs # = CMIE and † = HIIE.

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after SIE compared to baseline, and greater phosphorylation immediately after SIE compared to both HIIE and CMIE (Fig 3). Main time effects ($p < 0.05$) revealed greater phosphorylation of p38 MAPK immediately after exercise, and greater JNK phosphorylation immediately after and 3 hours after exercise compared to baseline (Fig 3). Main time effects ($p < 0.05$) revealed lower protein abundance of IκBα immediately and 3 hours after exercise compared to baseline (Fig 3).

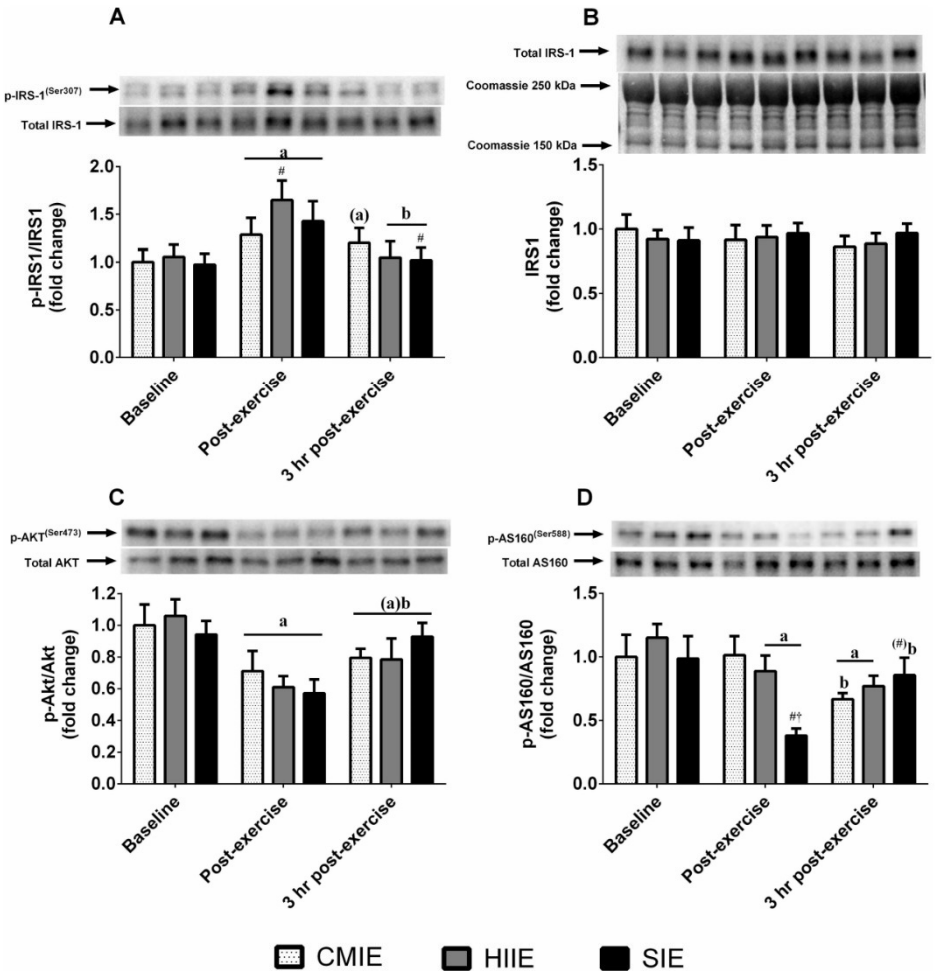

Fig 4. Skeletal muscle insulin protein signaling. Skeletal muscle total IRS-1 content **(B)** and phosphorylation relative to total protein content of **(A)** IRS-1^{Ser307}, **(C)** Akt^{Ser473}, and **(D)** AS160^{Ser588}, after high-intensity interval exercise **(HIIE)**, sprint interval exercise **(SIE)**, and continuous moderate-intensity exercise **(CMIE)**. a = $p < 0.05$ and (a) $p<0.1$ compared to baseline; b = $p < 0.05$ and (b) $p < 0.1$ compared to post-exercise. Significantly different ($p < 0.05$) or trend ($p < 0.1$ in parenthesis) at equivalent time point vs # = CMIE and † = HIIE.

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Skeletal muscle insulin protein signaling

A significant interaction effect ($p < 0.05$) was detected for IRS-1^{Ser307} phosphorylation. Post-hoc analysis revealed significantly greater IRS-1^{Ser307} phosphorylation immediately after all exercise bouts, and there was a trend for this to remain elevated at 3 hours after CMIE only (Fig 4). IRS-1^{Ser307} phosphorylation was significantly greater immediately after HIIE compared

to CMIE, and greater 3 hours after CMIE compared to SIE. A significant interaction effect ($p < 0.001$) was detected for $AS160^{Ser588}$ phosphorylation. Post-hoc analysis revealed lower phosphorylation of $AS160^{Ser588}$ immediately after SIE and HIIE compared to baseline, and 3 hours after CMIE and HIIE compared to baseline (Fig 4). $AS160^{Ser588}$ phosphorylation was lower immediately after SIE compared to HIIE and CMIE, and was higher 3 hours after SIE compared to CMIE. Phosphorylation of Akt^{Ser473} was lower immediately after exercise compared to baseline and tended to remain lower 3 hours after exercise (Fig 4). Despite increased IRS-1^{Ser307} phosphorylation, total IRS-1 protein was not significantly influenced by exercise (Fig 4).

Discussion

We report that a single session of SIE elicited greater skeletal muscle NF-κB p65 phosphorylation compared to HIIE and CMIE, a similar increase in JNK and p38 MAPK phosphorylation, and a similar decrease in skeletal muscle IκBα protein content. Thus, despite consisting of less total work than CMIE and HIIE, SIE proved to be an effective stimulus for the activation of stress protein kinase signaling pathways linked to exercise-mediated adaptation of skeletal muscle.

Exercise intensity and skeletal muscle SAPK signaling

NF-κB p65 phosphorylation in human skeletal muscle was increased immediately after SIE, but not after CMIE or HIIE. It is unclear why NF-κB p65 phosphorylation was not increased after CMIE or HIIE, as NF-κB activity/phosphorylation is increased in skeletal muscle of rodents after 1 hour of swimming and treadmill exercise [7, 28]. It is possible that only intense supramaximal exercise provides sufficient stimulus to increase NF-κB p65 phosphorylation in human skeletal muscle immediately after exercise. In support, Petersen *et al.* [29] reported no change in human skeletal muscle NF-κB p65 phosphorylation immediately after 45-minutes of continuous cycling (71% VO_{2peak}) or after cycling to exhaustion (92% VO_{2peak}). In addition to NF-κB p65 phosphorylation, transcriptional activity of NF-κB requires ubiquitin-dependent IκBα protein degradation, a process which permits inactive cytosolic NF-κB to translocate to the nucleus [30, 31]. Our findings align with others reporting decreased IκBα protein abundance in skeletal muscle after acute exercise [19, 29, 32]. This decrease appears to occur independent of NF-κB p65 phosphorylation and exercise-intensity. It is possible that our biopsy sampling times may not have captured peak NF-κB phosphorylation with CMIE and HIIE, which is increased one hour after HIIE in human skeletal muscle [19], and is reported to peak 1–2 hours after exercise in human PBMC [31] and rat skeletal muscle [33].

Attenuation of the exercise-induced skeletal muscle NF-κB p65 signaling response in humans and rodents, via allopurinol, apocynin, or n-acetylcysteine treatment/ingestion, coincides with attenuation of PGC-1α, manganese superoxide dismutase, glutathione peroxidase, citrate synthase, and mitochondrial transcription factor A gene expression [6, 7, 29]. As such, greater NF-κB p65 phosphorylation after acute SIE may contribute to the equivalent or superior skeletal muscle and cardiometabolic adaptations previously reported with SIE training [1].

The p38 MAPK and JNK signaling pathways play an important role in exercise-mediated mitochondrial biogenesis and antioxidant defense upregulation [34–38]. We provide evidence that JNK and p38 MAPK phosphorylation are increased to a similar extent after SIE, CMIE, and HIIE work-matched to CMIE. These findings support previous reports of similar post-exercise p38 MAPK phosphorylation after continuous exercise work-matched to high-intensity continuous cycling [39], HIIE [22], and SIE [23]. Furthermore, we showed that exercise-induced skeletal muscle JNK phosphorylation in humans does not appear to occur in an

exercise-intensity and/or volume manner, contradicting previous reports in rodents [40, 41]. Recently, Combes *et al.* [21] reported greater phosphorylation of p38 MAPK in human skeletal muscle with intermittent cycling (30 x 1-min intervals at 70% $VO2_{peak}$; 1-minute recovery periods) compared to work and intensity matched continuous cycling (30 minutes at 70% VO_{2peak}). It was proposed that increased oscillations of the cytosolic NADH/NAD+ redox state [42] elicited through intermittent exercise may play a larger role in p38 MAPK signaling compared to the manipulation of exercise volume or intensity. It is possible that the metabolic demands induced through HIIE and SIE in this and other studies were insufficient to increase p38 MAPK, and potentially JNK phosphorylation, above that of continuous exercise [22, 23]. Further research is required to confirm these findings with exercise protocols that incorporate greater metabolic disturbances.

The present findings suggest that superior skeletal muscle adaptation previously reported with HIIE and SIE when compared to CMIE [1, 2, 4, 5], may occur through protein signaling pathways independent of p38 MAPK and JNK. Nevertheless, SIE consisted of considerably less total work than HIIE and CMIE, and therefore appears to be an effective exercise mode for stimulating post-exercise skeletal muscle phosphorylation of p38 MAPK, JNK, and in particular NF-κB p65.

Exercise-intensity and phosphorylation of skeletal muscle insulin protein signaling

We provide evidence that IRS-1^{Ser307} phosphorylation is increased immediately after CMIE and SIE, and to a greater extent after HIIE. Interestingly, IRS-1^{Ser307} phosphorylation was similar to baseline 3 hours after HIIE and SIE. The physiological role of IRS-1^{Ser307} phosphorylation is unclear, as it is reported to both positively and negatively regulate downstream insulin signaling and glucose uptake [19, 43]. Akt^{Ser473} phosphorylation, which is downstream of IRS-1, decreased to a similar extent after all exercise protocols. Surprisingly, further probing of the distal insulin signaling cascade revealed that phosphorylation of $AS160^{Ser588}$ was attenuated in an exercise-intensity and post-exercise time-course dependent manner.

Phosphorylation of AS160 (also known as TBC1D4) results in GTP loading and activation of Rabs, releasing GLUT4 vesicles from intracellular compartments and promoting GLUT4 vesicle plasma membrane docking and glucose uptake [44]. Serine 588 specific phosphorylation of AS160 increases with human skeletal muscle contraction, insulin stimulation via the hyperinsulinaemic-euglycaemic clamp, and may play a role in the acute post-exercise enhancement of insulin sensitivity [17, 19, 45, 46]. Previous research is equivocal, with studies reporting no change [47, 48] or increased phosphorylation of $AS160^{Ser588}$ after exercise in both rodents and humans [17, 19, 49, 50]. We are the first to report decreased $AS160^{Ser588}$ phosphorylation immediately after SIE and HIIE. Using the PAS160 antibody, which primarily detects $AS160^{Thr642}$ but also $AS160^{Ser588}$ [51, 52], Treebak *et al.* [53] also reported a decrease in AS160 phosphorylation immediately after high-intensity continuous cycling exercise (20 minutes, 80% VO_{2peak}), whereas phosphorylation was unchanged immediately after CMIE (30 mins, ~67% VO_{2peak}). We extend previous findings by reporting that $AS160^{Ser588}$ phosphorylation is similar to baseline 3 hours after SIE, but remains lower after HIIE and CMIE.

The mechanism for the substantial decrease in $AS160^{Ser588}$ phosphorylation immediately after SIE is unclear. The reported elevation in blood glucose during and immediately after SIE, and to a lesser extent after HIIE, suggests a transient counter-regulatory hormonal response previously reported after higher-intensity exercise [54]. Certainly, resistance exercise and extreme muscle damaging exercise inhibit insulin protein signaling [55, 56], likely through mTOR inhibition of the PI3K signaling pathway [57]. However, mTOR signaling does not

appear to be activated following acute SIE [9]. Alternatively, excess ROS such as hydrogen peroxide may override the potentiation of insulin signaling through the inactivation of protein tyrosine phosphatases [58–60], by increasing JNK and NF-κB mediated inhibition of the PI3K/Akt signaling pathway [61, 62]. In TNF-α/NF-κB induced insulin resistant human myotubes, targeted interference of the NF-κB signaling pathway restores insulin stimulated AS160 and Akt phosphorylation and glucose uptake, despite minimal effect on JNK phosphorylation [13]. Taken together, it is possible that SIE induced NF-κB signaling may transiently suppress AS160 phosphorylation immediately after exercise. Whether the differential effect of exercise-intensity on post-exercise AS160$^{(Ser588)}$ phosphorylation occurs at other AS160 phosphorylation sites, and whether these changes effect post-exercise insulin sensitivity, are unknown and warrant further investigation.

Limitations

A potential limitation of the study is a small sample size. However, previous invasive human studies have used similar sample sizes to detect significant changes in SAPK signaling [21, 29, 63]. The combined analysis of both males and females may limit interpretation of the results. Nevertheless, exercise-induced p38 MAPK protein signaling appear to be similar between sexes [64]. Furthermore, in the current study we did not undertake subcellular fractionation, immunohistochemistry, and/or direct measurements of kinase activity due to limited tissue availability. Protein kinase signaling is reported to be spatial-temporally sensitive [30, 65] and as such future studies are required to determine the subcellular localization of protein kinase phosphorylation and kinase activity before and after exercise of different intensities and mode. It is also important to note that the acute activation of protein signaling pathways in skeletal muscle do not always reflect functional changes in protein synthesis and/or adaptations with chronic exercise training [23, 66]. Finally, findings in this study are delimited to young recreationally active adults, the specific exercise-protocols investigated, and the investigation of a single session of exercise. Future research is required to confirm these findings with subsequent bouts of exercise over a longer period of time, in more diverse populations with different exercise protocols.

Conclusions

These findings demonstrate that p38 MAPK and JNK phosphorylation increase to a similar extent after CMIE, HIIE and SIE. On the other hand, skeletal muscle NF-κB phosphorylation was more responsive to intense exercise. Whether greater NF-κB phosphorylation post-SIE contributes to the previously reported superior benefits of SIE on skeletal muscle adaption warrants further investigation. Surprisingly, only CMIE and HIIE elicited a decrease in phosphorylation of the downstream glucose uptake signaling protein AS160 three hours after exercise, despite substantially lower AS160 phosphorylation immediately after SIE. These findings indicate that the time course of post-exercise AS160 phosphorylation, an important regulator of contraction and insulin-stimulated glucose uptake, is influenced in an exercise-intensity dependent manner. Taken together, exercise-intensity plays a role in regulating the complex SAPK signaling pathways which are known to be involved in the adaptive cardiometabolic responses to exercise.

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Formal analysis: LP.

Funding acquisition: LP AT IL CSS NKS.

Investigation: LP AT IL CSS NKS.

Methodology: LP AT IL CSS NKS.

Project administration: LP AT IL CSS NKS.

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Supervision: IL CSS NKS.

Visualization: LP.

Writing – original draft: LP.

Writing – review & editing: LP AT IL CSS NKS.

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Acute High-Intensity Interval Exercise-Induced Redox Signaling Is Associated with Enhanced Insulin Sensitivity in Obese Middle-Aged Men

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Background: Obesity and aging are associated with increased oxidative stress, activation of stress and mitogen activated protein kinases (SAPK), and the development of insulin resistance and metabolic disease. In contrast, acute exercise also increases oxidative stress and SAPK signaling, yet is reported to enhance insulin sensitivity and reduce the risk of metabolic disease. This study explored this paradox by investigating the effect of a single session of high-intensity interval-exercise (HIIE) on redox status, muscle SAPK and insulin protein signaling in eleven middle-aged obese men.

Methods: Participants completed a 2 h hyperinsulinaemic-euglycaemic clamp at rest, and 60 min after HIIE (4 × 4 mins at 95% HR_{peak}; 2 min recovery periods), separated by 1–3 weeks.

Results: Irrespective of exercise-induced changes to redox status, insulin stimulation both at rest and after HIIE similarly increased plasma superoxide dismutase activity, plasma catalase activity, and skeletal muscle 4-HNE; and significantly decreased plasma TBARS and hydrogen peroxide. The SAPK signaling pathways of p38 MAPK, NF-κB p65, and JNK, and the distal insulin signaling protein $AS160^{Ser588}$, were activated with insulin stimulation at rest and to a greater extent with insulin stimulation after a prior bout of HIIE. Higher insulin sensitivity after HIIE was associated with higher insulin-stimulated SOD activity, JNK, p38 MAPK and NF-κB phosphorylation ($r = 0.63$, $r = 0.71$, $r = 0.72$, $r = 0.71$; $p < 0.05$, respectively).

Conclusion : These findings support a role for redox homeostasis and SAPK signaling in insulin-stimulated glucose uptake which may contribute to the enhancement of insulin sensitivity in obese men 3 h after HIIE.

Keywords: redox signaling, insulin sensitivity, obesity, oxidative stress, high-intensity exercise, stress kinase

INTRODUCTION

Obesity is a major risk factor for insulin resistance and type 2 diabetes. Regular physical activity improves glycemic control and as such is a key lifestyle goal for the prevention and management of obesity and type 2 diabetes (Malin and Braun, 2016). Even a single session of exercise can enhance insulin sensitivity in the hours after exercise (Frosig and Richter, 2009). However, the mechanisms by which exercise improves insulin sensitivity are not completely understood, but may include oxidation-reduction (redox) reactions and their inherent capacity to both impair and/or facilitate insulin signaling and insulin-stimulated glucose uptake (Tiganis, 2011).

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and endogenous antioxidant defenses (Radak et al., 2013). While modest increases in ROS are regulated by endogenous antioxidants, certain ROS-inducing stimuli, such as exercise, diet, age, and disease, may overpower these systems in favor of oxidative stress (Valko et al., 2007). Chronic systemic oxidative stress is associated with obesity, insulin resistance, and type 2 diabetes (Valko et al., 2007; Bashan et al., 2009). Oxidative stress-induced insulin resistance can occur through protein modification via both lipid peroxidation and the activation of stress and mitogen activated protein kinase signaling (SAPK) pathways (Tanti and Jager, 2009; Tiganis, 2011). Sustained activation of these redox-sensitive signaling pathways leads to inhibitory phosphorylation of the insulin receptor substrate 1 (IRS-1) at human serine residues 312 and 307, promoting IRS-1 degradation, impaired insulin signaling, and attenuation of insulin-stimulated glucose uptake (de Alvaro et al., 2004; Werner et al., 2004; Gual et al., 2005; Archuleta et al., 2009; Tanti and Jager, 2009; Pillon et al., 2012). The prevention of IRS-1 degradation through the inhibition of ROS and/or SAPK signaling has been shown to restore insulin signaling and insulin-stimulated glucose uptake (Somwar et al., 2000; Geiger et al., 2005; Tiganis, 2011; Pillon et al., 2012). Collectively, these studies suggest a pathological role of redox induced lipid peroxidation and SAPK signaling in aberrant insulin signaling and subsequent insulin resistance.

Paradoxically, ROS produced during and after muscular contraction also transiently activate SAPK signaling pathways and lipid peroxidation (Kramer and Goodyear, 2007), however during this period glucose uptake and insulin sensitivity are reported to be enhanced (Frosig and Richter, 2009). An accumulation of research now suggests that depending on the biological context, redox signaling is integral for optimal physiological functioning and adaptation to physiological stress (Radak et al., 2013). Redox-signaling may play an important role in contraction-induced (Sandström et al., 2006) and insulin-stimulated glucose uptake (Kim et al., 2006; Bashan et al., 2009; Loh et al., 2009; Trewin et al., 2015); whether these redox-signaling pathways are activated with insulin stimulation after a single session of exercise are unknown. High-intensity interval exercise (HIIE) is an effective exercise mode for improving glycemic control in clinical populations (Gibala et al., 2012; Liubaoerjijin et al., 2016), however the impact of acute HIIE on redox-sensitive protein signaling and insulin sensitivity in obese middle-aged males is unknown. The aim of this study was to test the hypothesis that a single session of HIIE would transiently increase oxidative stress and SAPK signaling and insulin signaling which may, at least in part, be related to post-exercise enhancement of insulin sensitivity in middle-aged, obese males.

Abbreviations: 4-HNE, 4-hydroxynonenal; AS160, akt substrate of 160 kDa; GSK-3, glycogen synthase kinase 3; HEC, hyperinsulinaemic-euglycaemic clamp; HIIE, high-intensity interval exercise; IL-6, interleukin 6; IRS-1, insulin receptor substrate 1; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; JNK, c-Jun N-terminal kinases; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PKC, protein kinase C; ROS, reactive oxygen species; SAPK, stress and mitogen activated protein kinase; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

MATERIALS AND METHODS

Participants

Eleven middle-aged (58.1 ± 2.2 years mean ± SEM, range 40–70 years), obese men (BMI = 33.1 ± 1.4 $kg \cdot m^{-2}$), without diabetes (fasting glucose: 5.3 ± 0.2 $mmol \cdot L^{-1}$ and $HbA1_C$: 5.6 ± 0.1%; 34 ± 1.1 mmol/mol), participated in the study (Levinger et al., 2014). Verbal and written explanations about the study were provided to participants prior to providing written consent. Exclusion criteria for participation included medications known to affect insulin secretion and/or insulin sensitivity; musculoskeletal or other conditions that prevent daily activity; and symptomatic or uncontrolled metabolic or cardiovascular disease. This study was approved by the Victoria University Human Research Ethics Committee and carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Screening and Preliminary Testing

Participants were asked to complete a symptom limited incremental cycle $\dot{V}O_{2peak}$ determination test as previously described (Levinger et al., 2007). Ventilatory expired gas (15 second averages) was collected from each participant and analyzed using a metabolic cart (Medgraphics, Cardio2 and CPX/D System, USA).

Study Design

Participants abstained from food (overnight fast), physical activity (72 h), and alcohol and caffeine consumption (24 h) prior to each trial day. To avoid glycogen depletion volunteers were provided dietary information and asked to consume approximately 300 g of carbohydrate 24 h prior to their first trial. This was recorded in a diet diary and replicated for their second trial. For the main experimental trial participants completed a 2 h hyperinsulinaemic-euglycaemic clamp (insulin clamp) at rest (rest trial), and 60 min after HIIE (exercise trial), separated by 1–3 weeks.

Rest Trial

Participants arrived in the morning following an overnight fast and a 2 h insulin clamp was performed to measure baseline

insulin sensitivity via methods previously reported (Hutchison et al., 2011; Stepto et al., 2013; Levinger et al., 2014). In brief, insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused at 40 $mU.m^{-2}$ per minute for 120 min inducing a stable state of hyperinsulinemia (Levinger et al., 2014). Concomitantly, exogenous glucose was infused at a rate necessary to maintain a stable blood glucose reading of ∼5 $mmol.l^{-1}$ which was assessed every 5 min during the insulin clamp with an automated analyzer (YSI 2300 STAT Plus Glucose & Lactate Analyzer, USA). Insulin sensitivity was calculated by averaging the glucose infusion rate (GIR, $mg{\cdot}kg^{-1}{\cdot}min^{-1}$) over the final 30 min of the insulin clamp and normalized to serum insulin levels (*M*-Value) (Howlett et al., 2008). Muscle biopsies and venous blood samples were taken at baseline and after the 2 h insulin clamp (∼3 h after exercise).

Exercise Trial

Approximately 1–3 weeks later participants arrived after an overnight fast and performed 30 min of HIIE on a Lode cycle ergometer (Corvial, Lode B.V., Groningen, The Netherlands). The HIIE included a 4 min warm-up at 50–60% HR_{peak}, followed by 4 × 4 min cycling bouts at 95% HR_{peak}, interspersed with 2 min active recovery at 50–60% HR_{peak}. The target HR for the exercise session was determined by the heart rate reserve method using the following formula: Exercise target HR = % of target intensity (HR_{peak}–HR_{rest}) + HR_{rest}. After the exercise bout participants underwent 1 h of passive recovery after which the 2 h insulin clamp was performed as per the previous trial.

Skeletal Muscle and Blood Sampling

Vastus lateralis muscle and venous blood samples were taken at baseline and after the insulin clamp in the rest trial; and at baseline, pre-insulin clamp (1 h post-exercise) and post-insulin clamp (∼3 h post-exercise) in the exercise trial. Muscle samples were obtained from the vastus lateralis under local anesthesia (xylocaine 1%) utilizing a Bergström needle with suction (Evans et al., 1982). The samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Venous blood was collected from an antecubital vein (contra-lateral to infusions) via an intravenous cannula and appropriate collection tubes. Blood samples were centrifuged at 3500 rpm for 15 min at 4°C and plasma subsequently aliquoted and stored at −80°C until analyzed.

Plasma Redox Status Analysis

Plasma thiobarbituric acid reactive substances (TBARS; Cayman Chemical, Ann Arbor, MI, USA), Catalase activity (Cayman Chemical, Ann Arbor, MI, USA), Superoxide Dismutase (SOD) activity (Cayman Chemical, Ann Arbor, MI, USA), and Hydrogen Peroxide (Amplex UltraRed assay; Molecular Probes, Eugene, Oregon, USA) were analyzed by a spectrophotometer (xMark microplate spectrophotometer, Bio-Rad Laboratories, Mississauga, ON, Canada) in duplicate as per the manufacturer's instructions. One unit of catalase activity is defined as the amount of enzyme required to cause the formation of 1.0 nmol of formaldehyde per minute at 25°C. One unit of SOD activity is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. TBARS is expressed in nmol/ml of malondialdehyde equivalents. Plasma hydrogen peroxide is expressed in nmol/ml. Intra-assay coefficients of variation were determined from each duplicate for all participants and resulted in a coefficient of variation of 1.05, 1.99, 4.91, and 1.88% for TBARS, SOD, catalase and hydrogen peroxide, respectively. Inter-assay coefficients of variation for assay standards were 0.65, 3.65, 2.30, 1.20, for TBARS, SOD, catalase, and hydrogen peroxide, respectively.

Skeletal Muscle Protein Analysis

Abundance of specific proteins in muscle samples were determined with all constituents present (i.e., no centrifugation) (Murphy and Lamb, 2013). Thirty cryosections of skeletal muscle (20 μm) were cut (Cryostat HM550, Thermo Scientific, Australia) and homogenized with 300 μl of homogenizing buffer (0.125 M TRIS-HCL (pH 6.8), 4% SDS, 10% Glycerol, 10 mM EGTA, and 0.1 M DTT). Samples were rotated for 1 h at room temperature, vortexed and frozen at −80°C. Samples were then thawed on ice and protein content was determined using the Red 660 Protein Assay (G-Biosciences, St. Louis, MO, USA) as per the manufacturer's instructions. Eight Microgram of protein was prepared in 3 μl of Bromophenol blue (1%), heated for 5 min at 95°C and separated by 7.5% Criterion™ TGX™ Pre-Cast Gels. The separated proteins were transferred to a membrane (Trans-Blot Turbo PVDF Transfer Pack, Bio-Rad, Richmond, CA) with a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with Tris-Buffered Saline-Tween (TBST: 0.1 M Tris Base, 1.5 M NaCl, 0.1% Tween-20) and 5% skim milk for 1 h and then washed (4 × 5 min) with TBST. Membranes were incubated at 4°C overnight with the following primary antibodies: Phospho-SAPK/JNK$^{Thr183/Tyr185}$ (Cell Signaling Technology; CST #9251), SAPK/JNK (CST #9252), phospho-p38 MAPK$^{Thr180/Tyr182}$ (CST #9211), p38 MAPK (CST #9212), phospho-NF-κB p65^{Ser536} (CST #3033), NF-κB p65 (CST #8242), IκBα (CST #4814), phospho-PKC δ/θ$^{Ser643/676}$ (CST #9376), phospho-IRS-1^{Ser307} (CST #2384), phospho-AS160^{Ser318} (CST #8619), phospho-AS160^{Ser588} (CST #8730), phospho-GSK-3α/β$^{Ser21/9}$ (CST #9331), AS160 (CST #2447), 4-Hydroxynonenal (Abcam, ab46545), and IRS-1 (Millipore, 06-248). After incubation, membranes were washed with TBST, incubated at room temperature (1 h) with horseradish peroxidase conjugated secondary antibody, re-washed and incubated in SuperSignal West Femto Maximum Sensitivity substrate for 5 min, and protein densitometry measured via ChemiDoc™ MP System (Bio-Rad) and Image Lab software (Bio-Rad). Membranes were then washed briefly in TBST, stained with 0.1% Brilliant Blue R-350 (Sigma Aldrich, USA) in 1:1 methanol/distilled water (dH_2O) solution for 3 min, de-stained in 1:5:4 ethanol/acetic acid/dH_2O solution for 40 s, and rinsed with dH_2O and air-dried for ∼1 h prior to imaging. Densitometry values are expressed relative to a pooled internal standard and normalized to the total protein content of each lane obtained from the modified Coomassie staining protocol. Where appropriate,

phosphorylated proteins are expressed relative to total specific protein content.

Statistical Analysis

Data were checked for normality and analyzed using Predictive Analytics Software (PASW, USA). Comparisons of means at baseline and after insulin stimulation in the rest and exercise trial were examined using a two way (intervention x time point) repeated measures analysis of variance (ANOVA). Comparisons of multiple means within the exercise trial were examined using a one way repeated measures ANOVA. *Post-hoc* analysis were conducted using Fisher's LSD. Associations between insulin sensitivity, redox status and SAPK signaling were analyzed using Pearson's coefficient of correlation. Cook's Distance was used as a measure of influence where observations greater than one Cook's D were excluded from correlation analysis (Cook, 1979). All data are reported as mean ± standard error of mean (SEM) and all statistical analysis were conducted at the 95% level of significance ($p \leq 0.05$). Trends were reported when p-values were greater than 0.05 and less than 0.1.

RESULTS

Insulin Sensitivity

Previously reported, exercise significantly increased insulin sensitivity (glucose infusion rate and m-value) by ∼34–40% compared to the rest trial (Levinger et al., 2014).

Plasma Redox Status

One hour after the HIIE session (prior to insulin stimulation) catalase activity significantly increased and TBARS and hydrogen peroxide decreased (**Figure 1**). Insulin stimulation both at rest and after exercise elicited a similar decrease in plasma hydrogen peroxide and TBARS, and increase in plasma catalase and superoxide dismutase activity (**Figure 1**).

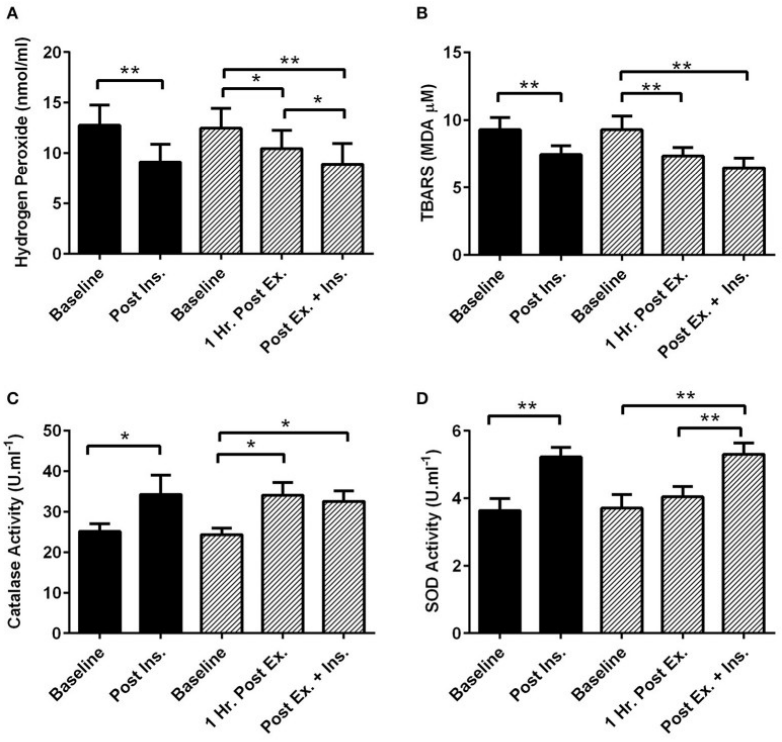

FIGURE 1 | Exercise, insulin stimulation, and plasma redox status. Oxidative stress and antioxidant enzymatic activity biomarkers in plasma 1 h after exercise (prior to insulin stimulation), and pre and post insulin stimulation at rest and 3 h after exercise ($n = 11$). **(A)** Hydrogen Peroxide; **(B)** Thiobarbituric acid reactive substances (TBARS); **(C)** Catalase activity; **(D)** Superoxide dismutase activity (SOD). $^{*}p < 0.05$ and $^{**}p < 0.01$ are significantly different. Black bars = rest trial. Diagonal line bars = exercise trial. Ex. = High-intensity interval exercise. Ins. = insulin stimulation via the hyperinsulinaemic-euglycaemic clamp.

Skeletal Muscle SAPK Signaling

The acute session of exercise (prior to insulin stimulation) significantly increased phosphorylation of JNK$^{Thr183/Tyr185}$, p38 MAPK$^{Thr180/Tyr182}$, NF-κB p65^{Ser536}, GSK-3α/β$^{Ser21/9}$, and 4-HNE protein modification (**Figures 2**, **3**). In contrast, phosphorylated PKC δ/θ$^{Ser643/676}$ was significantly lower after exercise (**Figure 3**). Insulin stimulation in the rest trial significantly increased phosphorylation of JNK$^{Thr183/Tyr185}$, p38 MAPK$^{Thr180/Tyr182}$, and 4-HNE protein modification (**Figure 2**). The prior bout of HIIE significantly increased insulin-stimulated phosphorylation of JNK, p38 MAPK, and NF-κB p65 to a greater extent. PKC δ/θ phosphorylation remained lower compared to baseline. Total protein content of IκBα was significantly lower after insulin stimulation in both the rest and HIIE trial (**Figure 2**). There was a tendency for increased phosphorylation of GSK-3α/β after insulin stimulation in both the rest and exercise trial.

Skeletal Muscle Insulin Signaling

Insulin stimulation both at rest and after HIIE elicited a similar increase in phosphorylation of IRS-1^{Ser307} and AS160^{Ser318} (**Figure 4**). There was a tendency for increased AS160^{Ser588} phosphorylation after HIIE (**Figure 4**). Insulin stimulated AS160^{Ser588} phosphorylation was greater after a prior bout of HIIE.

Insulin Sensitivity Correlations

Insulin sensitivity (*m*-value) following exercise was positively associated with higher levels of insulin-stimulated SOD activity ($r = 0.634$, $p = 0.036$, $n = 11$), phosphorylated-JNK ($r = 0.709$, $p = 0.007$, $n = 10$), p38 MAPK ($r = 0.724$, $p = 0.018$, $n = 10$) and NF-κB p65 ($r = 0.708$, $p = 0.022$, $n = 10$), and tended to correlate with lower levels of phosphorylated PKC δ/θ ($r = -0.571$, $p = 0.066$, $n = 11$). No correlations between insulin sensitivity and the variables of interest were detected in the rest trial ($p > 0.05$).

DISCUSSION

We report the novel finding that insulin-stimulated phosphorylation of p38 MAPK, NF-κB p65, and JNK occurred to a greater extent after a prior bout of HIIE in obese middle-aged males. Furthermore, increased SAPK signaling coincided with enhanced insulin signaling and whole body insulin sensitivity, indicating a potential role for SAPK signaling in the post-exercise enhancement of insulin sensitivity. We also reported that insulin stimulation increased plasma antioxidant activity, decreased plasma oxidative stress, and increased 4-HNE in skeletal muscle to a similar extent, irrespective of exercise-induced changes in redox status.

PRIOR EXERCISE, INSULIN STIMULATION, AND SAPK AND INSULIN SIGNALING

We report for the first time that in middle-aged obese males, p38 MAPK, NF-κB p65, and JNK signaling pathways are activated with insulin stimulation at rest, and to a greater extent with insulin stimulation after a prior bout of HIIE. Surprisingly, increased p38 MAPK, NF-κB p65, and JNK signaling did not coincide with greater IRS-1$^{(ser307)}$ phosphorylation, decreased phosphorylation of AS160$^{(ser318,\ ser588)}$, or impaired insulin sensitivity. Sustained activation of 4-HNE, NF-κB, JNK, p38 MAPK, and PKC signaling have been linked to attenuated insulin action and signaling via IRS-1 serine phosphorylation (de Alvaro et al., 2004; Gual et al., 2005; Archuleta et al., 2009; Pillon et al., 2012). In contrast, the transient activation of SAPK signaling pathways may augment insulin signaling and insulin-stimulated glucose uptake (Somwar et al., 2000; Thong et al., 2003; Geiger et al., 2005; Kim et al., 2006; Sandström et al., 2006; Berdichevsky et al., 2010). Our findings support redox sensitive SAPK signaling as a regulator of *in vivo* insulin-stimulated glucose uptake which may, at least in part, contribute to the post-exercise enhancement of insulin sensitivity.

We report similar insulin stimulated phosphorylation of IRS-1$^{(ser307)}$ both at rest and after a prior bout of HIIE. Insulin-stimulated phosphorylation of IRS-1^{ser307} may occur due to feedback inhibition of the insulin signaling cascade reported during conditions of hyperinsulinemia (Lee et al., 2003; Werner et al., 2004). Alternatively, IRS-1$^{(ser307)}$ phosphorylation may be necessary for insulin-stimulated propagation of the insulin signaling cascade and glucose uptake (Danielsson et al., 2005). Regardless of the biological role, IRS-1$^{(ser307)}$ phosphorylation was similar between rest and exercise trials and therefore unlikely to be contributing to the post-exercise enhancement of insulin sensitivity. These findings support previous reports that post-exercise enhancement of insulin sensitivity likely occurs downstream in the insulin signaling cascade (Frosig and Richter, 2009; Castorena et al., 2014).

p38 MAPK can be activated by both insulin and contraction of skeletal muscle, and may play a role in glucose metabolism (Somwar et al., 2000; Thong et al., 2003). Current cell culture and animal research is divided with research reporting a role for p38 MAPK in both insulin sensitivity (Somwar et al., 2000; Geiger et al., 2005) and insulin resistance (Diamond-Stanic et al., 2011). The discrepancy in findings may be the result of non-specific p38 MAPK inhibitors which can influence metabolism through modification of AKT, GSK-3 and AMPK signaling pathways independent of p38 MAPK (Kim et al., 2006; Diamond-Stanic et al., 2011). However, even when a specific p38 MAPK inhibitor was used phosphorylation/activity of p38 MAPK was either not measured or did not change under the conditions of interest (Diamond-Stanic et al., 2011). In humans, a recent study found a small but significant reduction (~6%) in post-exercise insulin sensitivity with the infusion of the antioxidant n-acetylcysteine (Trewin et al., 2015). Although p38 MAPK phosphorylation was decreased immediately after exercise with n-acetylcysteine infusion, phosphorylation 3 h after exercise and a further 2 h later after insulin stimulation were not different to baseline levels or the placebo intervention (Trewin et al., 2015). In contrast, Thong et al. (2003) reported p38 MAPK phosphorylation in human skeletal muscle to be increased 3 h after one-legged knee extensor exercise which was further phosphorylated after insulin stimulation. In support of the insulin sensitizing role of p38 MAPK in humans, we measured increased phosphorylation with insulin stimulation at rest, 1 h after exercise (prior to insulin

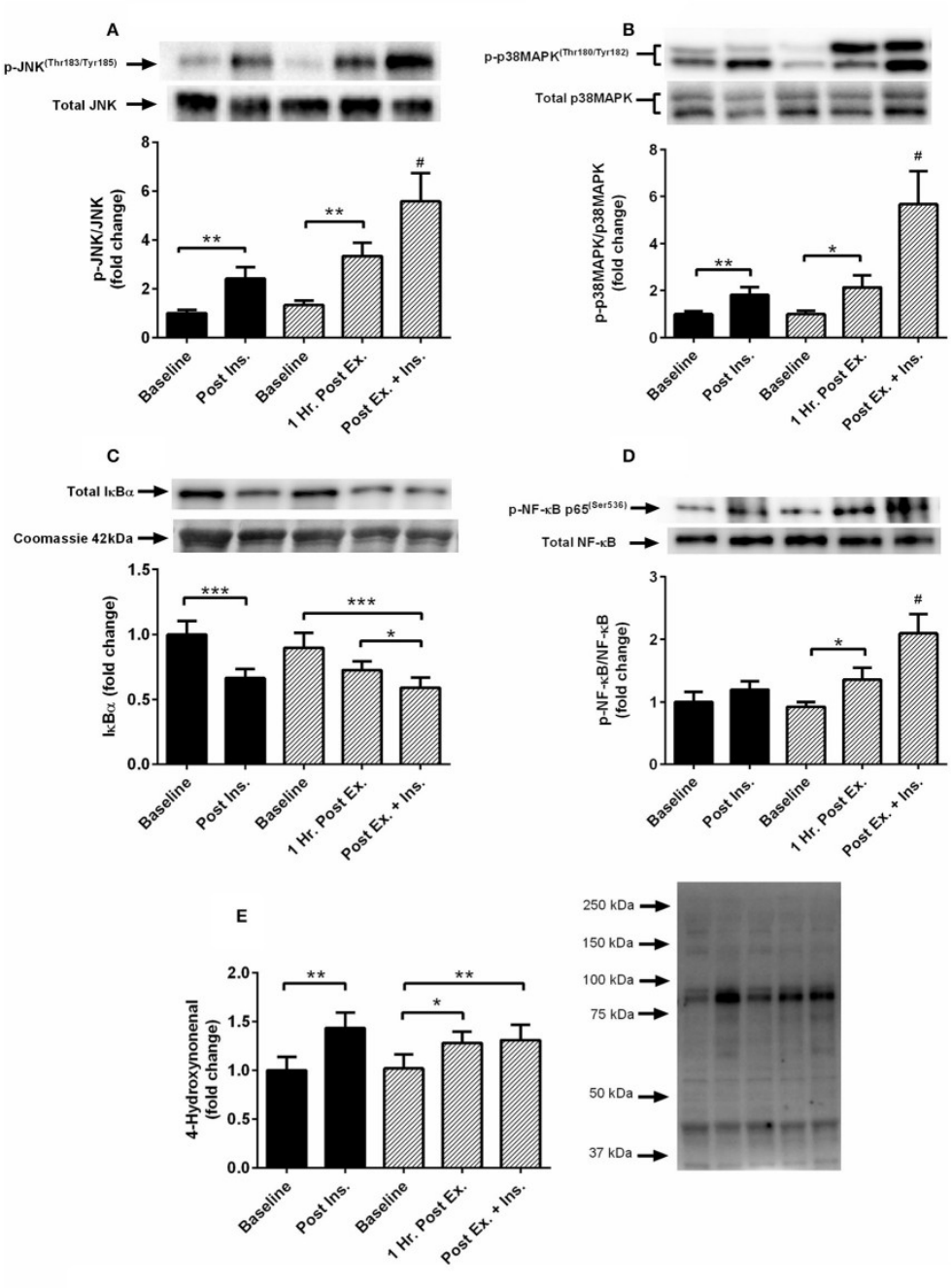

FIGURE 2 | Exercise, insulin stimulation, and skeletal muscle SAPK signaling. Phosphorylation relative to total protein content in skeletal muscle 1 h after exercise (prior to insulin stimulation), and pre and post insulin stimulation at rest and 3 h after exercise ($n = 11$). **(A)** JNK$^{(Thr183/Tyr185)}$; **(B)** p38 MAPK$^{(Thr180/Tyr182)}$; **(C)** IκBα; **(D)** NF-κB p65$^{(Ser536)}$; **(E)**: 4-hydroxynonenal. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and # is significantly different to all time-points in both the rest and exercise trial. Black bars = rest trial. Diagonal line bars = exercise trial. Ex. = High-intensity interval exercise. Ins. = insulin stimulation via the hyperinsulinaemic-euglycaemic clamp.

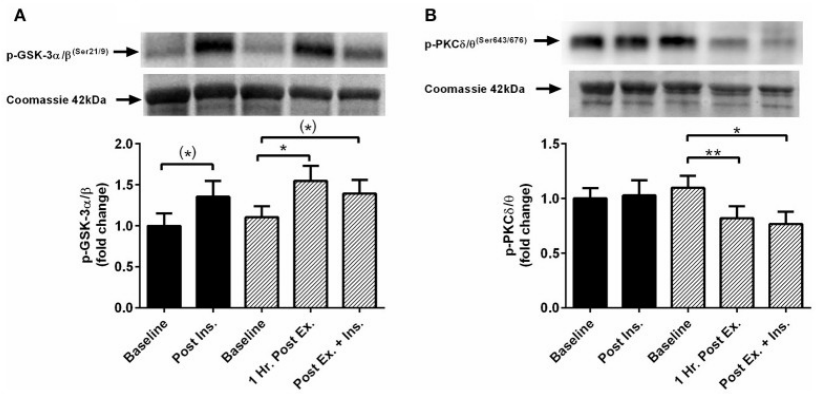

FIGURE 3 | Exercise, insulin stimulation, and skeletal muscle glycogen synthase kinase and protein kinase C signaling. Phosphorylation relative to total protein content in skeletal muscle 1 h after exercise (prior to insulin stimulation), and pre and post insulin stimulation at rest and 3 h after exercise ($n = 11$). **(A)** GSK-3α/β$^{(Ser21/9)}$; **(B)** PKC δ/θ$^{(Ser643/676)}$. $^{*}p < 0.05$ and $^{**}p < 0.01$ are significantly different. Black bars = rest trial. Diagonal line bars = exercise trial. Ex. = High-intensity interval exercise. Ins. = insulin stimulation via the hyperinsulinaemic-euglycaemic clamp.

stimulation), and the greatest phosphorylation with insulin stimulation after the prior bout of HIIE. The discrepancy in findings between Trewin et al. (2015) and ours may be due to differences in exercise intensity (55 min at 65%$\dot{V}O_{2peak}$ vs. HIIE), the participants' age (young vs. middle-aged), level of physical activity (recreationally active vs. inactive), body composition (BMI 24.8 vs. 33.1 kg·m^{-2}), and muscle biopsy sampling times.

The redox sensitive JNK pathway can be activated by acute exercise and has been suggested to play a role in insulin signaling and glucose metabolism (Ropelle et al., 2006; Berdichevsky et al., 2010; Tiganis, 2011). In contrast to the current findings, Castorena et al. (2014) reported enhanced insulin sensitivity 3 h after exercise in both high fat diet and low fat diet fed rats, despite minimal effect on JNK and IRS-1$^{(ser307)}$ phosphorylation. Furthermore, Ropelle et al. (2006) found that a single session of exercise in male rats reversed diet-induced insulin resistance 16 h later which coincided with decreased JNK, IκB–NF-κB, and IRS-1 serine phosphorylation. In support of the findings of the current study, Berdichevsky et al. (2010) reported that both chronic oxidative stress (1 μM of hydrogen peroxide for 48 h) and acute oxidative stress (500 μM of hydrogen peroxide for 3 h) similarly increased JNK phosphorylation in insulin resistant muscle cell lines. Remarkably, chronic oxidative stress increased IRS-1$^{(ser307)}$ phosphorylation and insulin resistance whereas acute oxidative stress rescued insulin sensitivity and insulin signaling through redistribution of active cytoplasmic JNK into the nucleus. The effect of JNK and NF-κB protein signaling on the enhancement and/or impairment of insulin sensitivity is likely spatial-temporal sensitive. The current findings support a potential insulin sensitizing role of exercise-induced JNK phosphorylation in overweight, middle-aged males, however further mechanistic research is required to confirm these findings.

In the absence of changes in proximal insulin signaling, exercise-induced SAPK signaling may influence insulin sensitivity through downstream insulin signaling events. Indeed, elevated ROS production caused by glutathione peroxidase-1 knockout mice results in increased phosphorylation of AKT$^{(Ser473)}$ and enhanced insulin sensitivity 60 min after treadmill exercise (Loh et al., 2009). Furthermore, acute hydrogen peroxide exposure (500 μM for 3 h) in C2C12 myoblasts increases insulin-stimulated glucose uptake alongside increased phosphorylation of JNK, AKT$^{(Ser473)}$, AKT$^{(Thr308)}$, and decreased GSK3-α/β activity (Berdichevsky et al., 2010). In contrast, isolated skeletal muscle of lean Zucker rats incubated in hydrogen peroxide (90 μM for 2 h) is reported to increase p38 MAPK phosphorylation while concomitantly decreasing AKT$^{(Ser473)}$ phosphorylation and insulin sensitivity (Dokken et al., 2008). In the present study, we found increased insulin-stimulated SAPK signaling after exercise to coincide with enhanced insulin sensitivity and increased phosphorylation of AS160$^{(Ser588)}$ (Levinger et al., 2014). Modulation of glycogen synthesis by oxidative stress-induced SAPK signaling is also suggested to mediate glucose metabolism (Dokken et al., 2008; Berdichevsky et al., 2010). However, similar insulin-stimulated GSK-3α/β$^{(Ser21/9)}$ phosphorylation at rest and post-HIIE suggests this as an unlikely pathway for post-exercise enhancement of insulin sensitivity.

Protein kinase C has multiple isoforms which are suggested to play a role in insulin-stimulated glucose uptake (Frosig and Richter, 2009; Tiganis, 2011). The novel PKC δ/θ isoforms are sensitive to both change in redox status and the concentration of the lipid intermediate diacylglycerol (Ragheb et al., 2009). PKC δ/θ activation impairs insulin signaling through serine phosphorylation of IRS-1 (Greene et al., 2004; Li et al., 2004). Interestingly, phosphorylated PKC δ/θ was attenuated 1 h after HIIE and continued to be so 3 h later after insulin stimulation. This may be clinically important as obese individuals exhibit elevated levels of plasma free

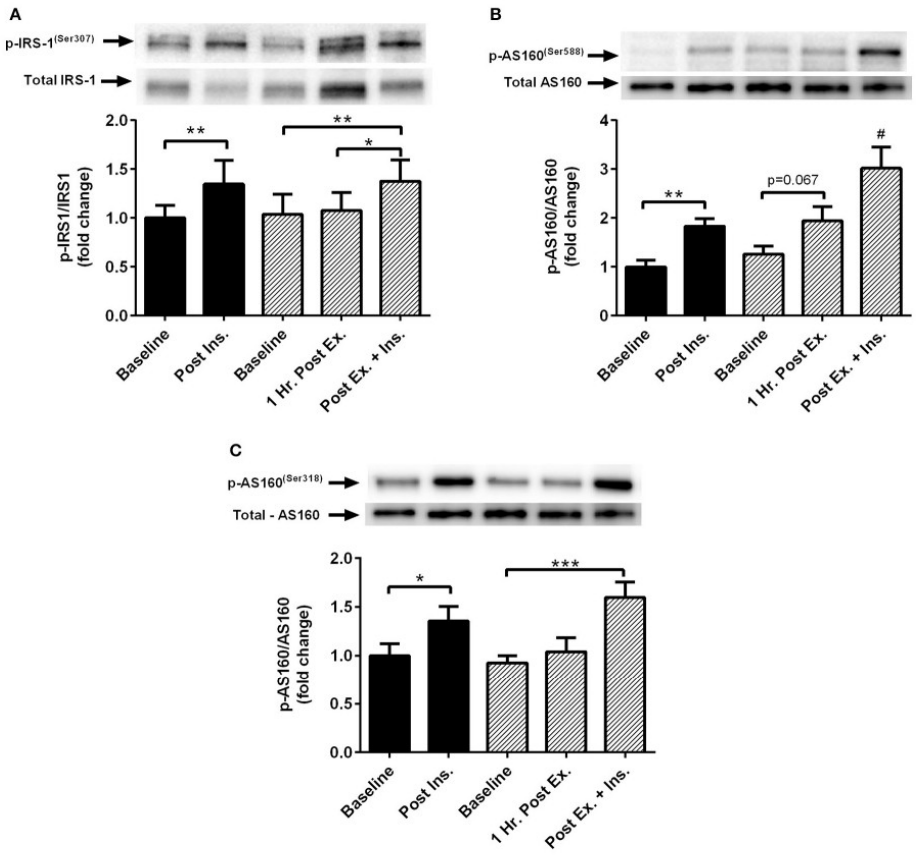

FIGURE 4 | Exercise, insulin stimulation and skeletal muscle insulin signaling. Phosphorylation relative to total protein content of insulin signaling proteins in skeletal muscle 1 h after exercise (prior to insulin stimulation), and pre and post insulin stimulation at rest and 3 h after exercise ($n = 11$). **(A)** IRS-1$^{(ser307)}$; **(B)** AS160$^{(ser588)}$; **(C)** AS160$^{(ser318)}$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and # is significantly different to all time-points in both the rest and exercise trial. Black bars = rest trial. Diagonal line bars = exercise trial. Ex. = High-intensity interval exercise. Ins. = insulin stimulation via the hyperinsulinaemic-euglycaemic clamp.

fatty acids (Boden, 2008) and increased PKC activity in response to insulin (Li et al., 2004). Indeed, lower insulin-stimulated PKC δ/θ phosphorylation in the exercise trial tended to correlate with higher insulin sensitivity, however phosphorylated IRS-1$^{(Ser307)}$ was similar between the rest and exercise trial. SAPK signaling, PKC δ/θ activity, and insulin resistance often occur concomitantly (Ragheb et al., 2009). In the present study, SAPK signaling increased, PKC δ/θ phosphorylation decreased, and insulin sensitivity was enhanced after HIIE, suggesting independent pathways for PKC δ/θ and SAPK phosphorylation post-HIIE. Thus, PKC δ/θ phosphorylation may be a good candidate for further investigation with respect to post-HIIE enhanced insulin sensitivity in humans.

REDOX STATUS AND INSULIN SIGNALING

The important role of redox biology in promoting and/or attenuating insulin sensitivity is well established in non-human models (Tiganis, 2011). We provide evidence that insulin stimulation increases plasma antioxidant capacity, decreases plasma oxidative stress, and increases 4-HNE in human skeletal muscle. Furthermore, this insulin stimulated shift in redox status also occurred with insulin stimulation after acute HIIE. Interestingly, the redox shift elicited by exercise (prior to insulin stimulation) had minimal effect on insulin stimulated redox status. Mahadev et al. (2004) reported that insulin stimulation increases hydrogen peroxide via increased NADPH oxidase activity, promoting insulin signaling and glucose uptake in part

via decreased protein tyrosine phosphatase activity. Furthermore, previous research has shown that hydrogen peroxide can both attenuate and/or enhance insulin-stimulated glucose uptake *in-vitro* depending on its concentration (Iwakami et al., 2011). It is possible that redox status may be regulated by endogenous antioxidant defenses to maintain a redox environment conducive for optimal insulin signaling and glucose uptake. Indeed, alteration of redox homeostasis through exogenous antioxidants in humans are reported to attenuate the benefits of both acute and regular exercise on insulin sensitivity (Ristow et al., 2009; Trewin et al., 2015). We provide novel *in vivo* evidence in humans to support the important role of ROS and redox homeostasis in insulin-stimulated glucose uptake (Bashan et al., 2009; Tiganis, 2011).

ACUTE EXERCISE AND REDOX STATUS

A single session of HIIE increased plasma catalase activity and decreased plasma TBARS and hydrogen peroxide in obese middle-aged males. These findings contradict previous reports of increased systemic oxidative stress in obese individuals (Vincent et al., 2004, 2005). The discrepancy in findings are unclear, but may relate to increased antioxidant activity after HIIE, whereas previous studies have reported either no change or decreased antioxidant defense after continuous aerobic exercise (Vincent et al., 2004, 2005). Certainly, we and others have previously shown that higher-intensity exercise can elicit greater plasma antioxidant defense in untrained healthy males without incurring changes in plasma oxidative stress (Schneider et al., 2005; Parker et al., 2014). Obesity and aging is associated with higher levels of systemic oxidative stress which over time causes oxidative damage to proteins, lipids, and DNA, and the development of numerous pathological conditions (Valko et al., 2007). Transient shifts in redox homeostasis with regular exercise leads to the upregulation of antioxidant defense, reduces chronic oxidative stress and inflammation, and improves overall metabolic health (Ristow et al., 2009; Malin and Braun, 2016). We provide evidence that regular HIIE may be a beneficial exercise model for improving redox status and metabolic health in clinical populations.

ROS are beneficial and a necessary requirement for optimal physiological functioning and adaptation to exercise (Radak et al., 2013). We found 4-HNE protein modification, a marker of oxidative stress, to be increased in skeletal muscle 1 h after HIIE. This contradictory redox shift between plasma (decreased oxidative stress) and skeletal muscle (increased oxidative stress) may reflect the inability of plasma to accurately reflect skeletal muscle redox status (Veskoukis et al., 2009). Increased oxidative stress in skeletal muscle likely reflects localized cellular stress associated with muscular contraction and supports the important signaling role of ROS in the adaptation to exercise (Radak et al., 2013). Indeed, phosphorylation of redox-sensitive signaling proteins JNK, p38 MAPK, and NF-κB were significantly increased one after HIIE. Activation of these pathways with exercise is known to promote improvements in redox homeostasis, regulation of energy metabolism, muscle hypertrophy, inflammation, and gene transcription leading to cell proliferation, differentiation, and apoptosis (Kramer and Goodyear, 2007; Egan and Zierath, 2013). Our findings indicate that acute HIIE can transiently shift systemic redox status and activate redox-signaling pathways in skeletal muscle involved in adaptation to exercise in obese middle-aged men.

A potential limitation of the study is the small sample size. However, the present study was adequately powered to detect changes in insulin sensitivity and p38 MAPK phosphorylation in human skeletal muscle (Thong et al., 2003; Trewin et al., 2015). Additionally, only a single marker of oxidative stress was used to determine redox status in skeletal muscle. Measurement of multiple redox markers in skeletal muscle would allow for greater interpretation of the influence of skeletal muscle redox status under the conditions of interest. This study is limited to the measurement of muscle and plasma responses to a single session of HIIE and insulin stimulation. Future research would benefit by investigating these findings with respect to subsequent bouts of exercise over a longer period of time. The inclusion of an exercise only control trial would allow for greater understanding of the combined effects of exercise and insulin stimulation on the measured outcomes. Data presented in this study are delimited to obese middle-aged males and the specific HIIE protocol used. Further research is required to confirm these findings in other populations and different exercise protocols.

CONCLUSION

In summary, we provide evidence that redox status and SAPK signaling are affected by both insulin stimulation and a single session of HIIE in obese middle-aged males. A prior bout of HIIE elicited greater insulin stimulated JNK, p38 MAPK, and NF-κB signaling which coincided with enhanced distal insulin signaling and whole body insulin sensitivity. These findings support the role of SAPK signaling in glycemic control and provide potential signaling pathways for the post-exercise enhancement of insulin sensitivity. Future research is required to explore potential mechanisms.

CLINICAL TRIAL NUMBER

ACTRN12613000706774, anzctr.org.au.

AUTHOR CONTRIBUTIONS

LP, IL, CS, NS, DH, and MA contributed to the study design and acquirement of ethical approval. LP, IL, NS, FS and MA contributed to data collection. LP analyzed the data, interpreted the data, and drafted the initial manuscript. The remaining authors critically revised the manuscript. All authors approved the final version of the manuscript. IL and LP are guarantors of the manuscript and take full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

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The Effect of Acute Exercise on Undercarboxylated Osteocalcin and Insulin Sensitivity in Obese Men

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ABSTRACT

Acute exercise improves insulin sensitivity for hours after the exercise is ceased. The skeleton contributes to glucose metabolism and insulin sensitivity via osteocalcin (OC) in its undercarboxylated (ucOC) form in mice. We tested the hypothesis that insulin sensitivity over the hours after exercise is associated with circulating levels of ucOC. Eleven middle-aged (58.1 ± 2.2 years mean ± SEM), obese (body mass index [BMI] = 33.1 ± 1.4 kg/m^2) nondiabetic men completed a euglycemic-hyperinsulinemic clamp at rest (rest-control) and at 60 minutes after exercise (4 × 4 minutes of cycling at 95% of HR_{peak}). Insulin sensitivity was determined by glucose infusion rate relative to body mass (GIR, mL/kg/min) as well as GIR per unit of insulin (M-value). Blood samples and five muscle biopsies were obtained; two at the resting-control session, one before and one after clamping, and three in the exercise session, at rest, 60 minutes after exercise, and after the clamp. Exercise increased serum ucOC (6.4 ± 2.1%, $p = 0.013$) but not total OC ($p > 0.05$). Blood glucose was ~6% lower and insulin sensitivity was ~35% higher after exercise compared with control (both $p < 0.05$). Phosphorylated (P)-AKT (Ak thymoma) was higher after exercise and insulin compared with exercise alone (no insulin) and insulin alone (no exercise, all $p < 0.05$). In a multiple-linear regression including BMI, age, and aerobic fitness, ucOC was associated with whole-body insulin sensitivity at rest ($\beta = 0.59$, $p = 0.023$) and after exercise ($\beta = 0.66$, $p = 0.005$). Insulin sensitivity, after acute exercise, is associated with circulating levels of ucOC in obese men. Whether ucOC has a direct effect on skeletal muscle insulin sensitivity after exercise is yet to be determined. © 2014 American Society for Bone and Mineral Research.

KEY WORDS: BONE METABOLISM; EXERCISE; GLYCEMIC CONTROL; OBESITY; UNDERCARBOXYLATED OSTEOCALCIN

Introduction

Exercise is important for the prevention and management of type 2 diabetes (T2DM). Even a single bout of exercise increases insulin sensitivity for hours after exercise is ceased.[1,2] This increase in sensitivity occurs in skeletal muscle, a major site for glucose disposal. Glucose disposal rate increases during and after exercise, even in those with elevated body mass and poorer glycemic control.[3] However, the factors responsible for this increase in insulin sensitivity after exercise are not completely understood.[4]

Recent work suggests that the skeleton is an endocrine organ that participates in glucose homeostasis.[5,6] Osteocalcin (OC) is an osteoblast-specific product that is secreted into the bone extracellular matrix and the general circulation and reflects bone remodeling.[7] In murine models, the undercarboxylated form of OC (ucOC) stimulates β-cell proliferation and insulin secretion, and ucOC-deficient mice are obese, glucose intolerant, and have features of T2DM.[6] In humans, ucOC, and OC, are also correlated with insulin sensitivity, fasting glucose, fat mass, and muscle strength.[8–11] Moreover, serum ucOC is associated with improved glucose tolerance in middle-aged men, an effect that may be related to enhanced β-cell function.[12] In addition, acute exercise increases ucOC, and this increase in ucOC is related to a reduction in serum glucose levels in obese men.[13] It is unclear whether increases in ucOC are related with improvement in whole-body insulin sensitivity after exercise or whether serum ucOC levels predict changes in skeletal muscle insulin signaling, including AKT and AS160. Akt and its 160-kDa substrate (AS160) are downstream proteins in the phosphatidylinositol 3-kinase (PI3-K) pathway and may be a convergence point between the pathways regulating insulin- and contraction-stimulated GLUT-4 translocation.[14,15]

The aim of this study was to test the hypothesis that in obese men, insulin sensitivity after exercise is related to the circulating levels of ucOC.

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Materials and Methods

Participants

Eleven middle-aged (58.1 ± 2.2 years mean ± SEM, range 40 to 70 years), obese (body mass index [BMI] = 33.1 ± 1.4 kg/m^2) nondiabetic men participated in the study (Table 1). Power and precision software was used to calculate the needed sample size (power of 80%, alpha = 0.05) to detect changes (>6%) in serum ucOC after acute aerobic exercise.[13] We excluded men with bone disease; men taking antihyperglycemic medications or medications known to affect bone metabolism, insulin secretion or insulin sensitivity, and musculoskeletal or other conditions that prevent daily activity; and men with symptomatic or uncontrolled metabolic or cardiovascular disease or receiving warfarin or vitamin K supplementation.

Each participant was given written and verbal explanations about the study before signing an informed consent form. The study protocol was approved by the Human Research Ethics Committee, Victoria University.

Study protocol

Participants underwent anthropometric measurements, assessment of their aerobic power (VO_{2peak}), and a fasting blood test. Participants also completed two sessions of 2-hour euglycemic-hyperinsulinemic clamp (insulin clamp), once at rest (rest-control) and once commenced 60 minutes after acute bout of exercise (after exercise). During the two insulin clamp sessions, five muscle biopsies were obtained from each participant (Fig. 1).

A blood sample was collected after overnight fast. Blood was analyzed at Austin Health (Melbourne, Australia) pathology using the standard hospital assay protocols for triglyceride, high-density lipoprotein (HDL), glucose, HbA1c, and insulin.

Blood pressure was measured using a standard mercury sphygmomanometer after the participant had rested in a seated position for at least 15 minutes.Weight was measured using a scale (TANITA, Tanita Corporation, Tokyo, Japan).

Aerobic power (VO_{2peak}) was assessed during a sign and symptom-limited graded exercise test as described.[16] VO_2 for each 15-second interval was measured by gas analysis (Medgraphics, Cardio2, and CPX/D System with Breezeex Software, 142090-001, Revia, MN, USA) that was calibrated before each test.

Table 1. Characteristics of Participants (Mean ± SEM)

	Mean ± SEM
Age (years)	58.1 ± 2.2
Height (cm)	176 ± 1.7
Mass (kg)	102.5 ± 3.9
BMI (kg/m^2)	33.1 ± 1.4
Fasting glucose (mmol/L)	5.3 ± 0.2
HbA1c (%)	5.6 ± 0.1
Trig (mmol/L)	1.9 ± 0.5
HDL (mmol/L)	1.3 ± 0.1
SBP (mmHg)	133.4 ± 3.6
DBP (mmHg)	84.5 ± 1.8
25OHD (nmol/L)	72.9 ± 7.6
VO_{2peak} (mL/kg/min)	23.0 ± 1.3
VO_{2peak} (mL/min)	2342 ± 160.8

BMI = body mass index; HbA1c = glycosylated haemoglobin; Trig = triglyceride; HDL = high-density lipoprotein; SBP = systolic blood pressure; DBP = diastolic blood pressure.

Experimental sessions

A diagram of the two experimental sessions is illustrated in Fig. 1. Participants attended our laboratory twice for the experimental trials, at 8 a.m. after an overnight fast. In the day before the experiment, volunteers were asked to consume around 300 g of carbohydrate to avoid glycogen depletion. The two experimental trials (control-rest or exercise) were conducted 3 to 5 weeks apart.

The control-rest session was to determine the basal (rest) insulin sensitivity as well as resting levels of OC and ucOC. Euglycemic-hyperinsulinemic clamp (insulin clamp) was performed at rest, as was reported previously.[17,18] Briefly, the clamps were performed after an overnight fast. Venous blood samples, heated arm vein, were collected before and during each session. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused at 40 mU/m^2 per minute for 120 minutes, generating an elevated, stable insulin concentration in the last 30 minutes of both sessions (rest-control: 70.5 ± 7.4 mIU/L and exercise 67.1 ± 6.2 mIU/L, $p < 0.001$ compared with baseline levels), confirming a hyperinsulinemic state (>24.9 μU/mL). Insulin sensitivity was assessed by the glucose infusion rate (GIR, mg/kg/min) during the last 30 minutes of the insulin-stimulated period and the GIR per unit of insulin (M-value).[19] During both hyperinsulinemic-euglycemic clamps sessions, exogenous glucose was variably infused to achieve the target blood glucose of ~5 mmol/L for the duration of the clamp, using variable infusion. Glucose was assessed every 5 minutes during the clamp (YSI 2300 STAT Plus Glucose & Lactate Analyzer, YSI, Inc., Yellow Springs, OH, USA). Two muscle biopsies were taken during this session, one before and one after the insulin clamp.

In the second session, an insulin clamp after an acute high-intensity bout of exercise was performed. Participants were supine, and a resting blood sample and resting muscle biopsy were taken. After the initial blood sampling and muscle biopsy, participants performed 30 minutes of high-intensity interval exercise that included one warm-up set of 4 minutes of exercise at approximately 50% to 60% of HR_{peak} followed by 4 sets (bouts) of 4 minutes each at 90% to 95% of HR_{peak}. The high-intensity intervals were separated by 2 minutes of "active" recovery (cycling at a lower intensity (50% to 60% of peak). Blood samples were obtained immediately after exercise and at 30 and 60 minutes after exercise to identify the peak change in OC and ucOC, glucose, and insulin. The insulin clamp commenced 60 minutes after exercise, and it was performed as described in session 1. Additional muscle biopsies were taken before and after the insulin clamp. Overall, three muscle biopsies were taken during session 2, at rest, 60 minutes after exercise and after insulin clamp.

Muscle biopsies

Muscle samples were obtained from the vastus lateralis under local anesthesia (Xylocaine 1%), utilizing the percutaneous needle biopsy technique with suction.[20] The samples were immediately frozen in liquid nitrogen and were then stored at −80°C until analysis.

Serum osteocalcin and undercarboxylated osteocalcin

Total serum OC was measured using an automated immunoassay (Elecsys 170; Roche Diagnostics, Mannheim, Germany). This assay has a sensitivity of 0.5 μg/L, with an intra-assay precision of 1.3%. Serum ucOC was measured by the same immunoassay

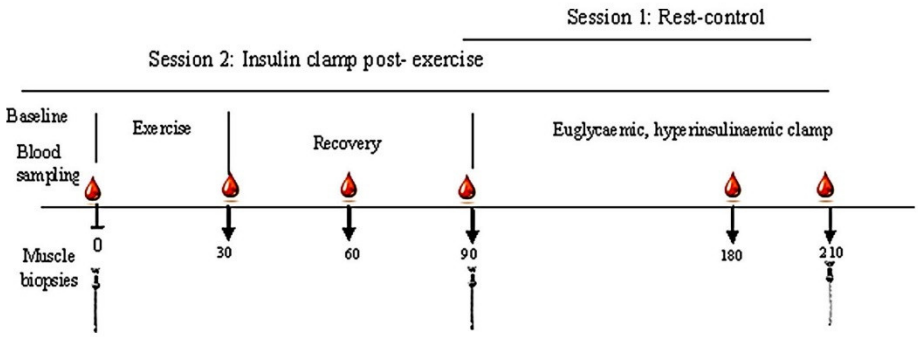

Fig. 1. Experimental design. Two muscle biopsies were obtained during the rest-control session (before and after insulin clamp), and three muscle biopsies were obtained during the exercise session (at baseline and before and after insulin clamp).

after adsorption of carboxylated OC on 5 mg/mL hydroxylapatite slurry, following the method described by Gundberg and colleagues.[21] The ucOC values on these samples of 6.0 to 35.6 μg/L and % unbound osteocalcin between 38.1% to 51.4% are in keeping with the original assay validation. The interassay coefficient of variation (CV) for total OC is 8.3% and the interassay CV for ucOC is 5.7%.

Western blots

Three to 4 mg of freeze-dried skeletal muscle was homogenized with 150 μL/mg of homogenizing buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 20 mM Tris, 1 mM EDTA, 5 mM Na pyrophosphate, 10 mM NaF, 1% Triton X-100, 10% glycerol, 200 μM PMSF, 0.5 mM Na4VO3, 1 mM benzamidine, and 0.1% protease inhibitor cocktail). Samples were rotated for 1 hour at 4°C, centrifuged at 15,000*g* (4°C) for 15 minutes, and the supernatant was collected. Protein content was determined using the Bradford Assay (Bio-Rad Laboratories, Hercules, CA, USA) per the manufacturer's instructions. All samples were diluted to a standard concentration of 2 μg/μL using 4× Laemmli buffer (containing 8 μL β-mercaptoethanol per 100 μL of Laemmli buffer) and homogenizing buffer. Total protein (25 μg) of each sample was loaded onto 7.5% Criterion TGX Gels and run for 45 minutes at 200 V. Samples were electroblotted (Criterion Blotter with Wire Electrodes, Bio-Rad) onto PVDF (0.45-mm pore) membrane in TOWBIN transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS, and pH 8.3) for 80 minutes at 100 V. Membranes were blocked with Tris-Buffered Saline-Tween (TBST: 0.1 M Tris Base, 1.5 M NaCl, 0.1% Tween-20) and 5% skim milk for 1 hour and then washed (5 × 8 minutes) with TBST. Membranes were incubated at 4°C overnight with the following primary antibodies: Phospho-Akt (Ser473), Akt, AS160, Phospho-AS160 (Thr642) (Cell Signaling Technology, Danvers, MA, USA), and α-tubulin (Abcam, Cambridge, UK). After incubation, membranes were washed with TBST, incubated for 1 hour at room temperature with appropriate dilutions of horseradish peroxidase-conjugated secondary antibodies, rewashed, and then exposed to Clarity Western ECL Substrate (Bio-Rad) for 1 minute. Membranes were scanned using a VersaDoc Imaging System (Bio-Rad) and densitometry analyzed using Quantity One software (Bio-Rad). All densitometry values were then expressed relative to a pooled internal standard and a corresponding α-tubulin loading control from the equivalent sample lysate.

Statistical analyses

Changes from before to after exercise within each trial and between trials were analyzed by paired *t* tests. The insulin signaling proteins AKT and AS160 (total and phosphorylated) were analyzed using general linear model ANOVA with Bonferroni correction. Multilinear regression model with ucOC, age, BMI, and aerobic fitness were used to determine association with insulin sensitivity. All data are reported as mean ± standard error of mean (SEM), and all statistical analyses were conducted at the 95% level of significance ($p \leq 0.05$).

Results

The characteristics of participants are shown in Table 1. Blood glucose, insulin, tOC, and ucOC were similar at baseline between the rest-control and exercise sessions (all $p > 0.05$, Table 2). At rest, in a multilinear regression model that included ucOC, BMI, age, and aerobic fitness has shown that both ucOC and BMI, but not age or aerobic fitness, were associated with resting insulin sensitivity (GIR, mg/kg/min, $\beta = 0.59$, $p = 0.023$, $\beta = -0.89$ $p = 0.003$, respectively).

Effects of exercise

Exercise intensity during the session was 95.1 ± 1.9% of HR_{peak}. The mean rate of perceived exertion was 16.3 ± 0.4 (rating range 6 to 20). Exercise increased the circulating ucOC (10.6 ± 0.8 to 11.2 ng/mL, $p < 0.05$) and ucOC/OC ratio (58.9 ± 2.0 to 62.1 ± 1.9%, $p = 0.023$) but not tOC ($p > 0.05$, Fig. 2). Blood glucose decreased (from 5.3 ± 0.3 to 5.0 ± 0.3, $p < 0.05$, Fig. 3)

Table 2. Baseline Comparisons Between the Rest-Control Session and the Exercise Session

	Rest-control	Exercise
Glucose (mmol/L)	5.2 ± 0.2	5.3 ± 0.3
Insulin (μU/mL)	13.1 ± 2.4	12.4 ± 2.4
tOC (ng/mL)	17.5 ± 1.4	18.2 ± 1.4
ucOC (ng/mL)	10.8 ± 0.8	10.6 ± 0.8
ucOC/OC ratio	60.8 ± 1.7	58.9 ± 2.0

tOC = total osteocalcin; ucOC = undercarboxylated osteocalcin.
Data presented as mean ± SEM. All comparisons $p > 0.05$.

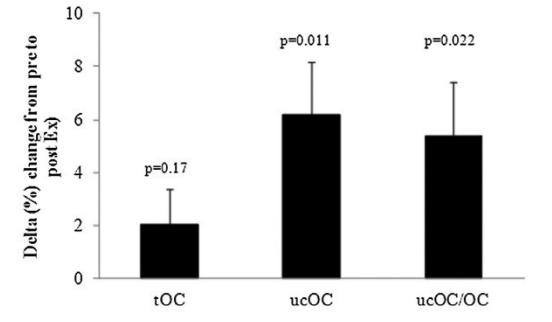

Fig. 2. Acute high-intensity aerobic exercise increases undercarboxylated OC (ucOC) and ucOC/OC ratio but not total osteocalcin (tOC).

during the 60 minutes after exercise, without change in serum insulin (13.8 ± 1.9 to 12.4 ± 2.4 mIU/L, $p = 0.33$).

The glucose infusion rate (GIR, mg/kg/min) needed to maintain blood glucose levels at ~5 mmol/L and the GIR per unit of circulating insulin (M-value), both measures of insulin sensitivity, were higher 3 hours after exercise compared with the rest-control session ($p < 0.05$, Fig. 3).

There was no significant change in total AKT (Fig. 4*A*) and total AS160 (Fig. 4*C*) after any treatment (after a resting insulin clamp, after exercise alone, and after exercise and insulin clamp $p > 0.05$). At rest, p-AKT, the active form of AKT, did not significantly change with insulin (rest-control session). However, after exercise and insulin (insulin clamp), p-AKT was significantly higher compared with exercise alone (no insulin, $p = 0.004$, Fig. 4*B*) and insulin alone (no exercise, $p = 0.029$, Fig. 4*B*). P-AS160 was higher after exercise and after exercise and insulin compared with the baseline level (Fig. 4*D*).

In a multilinear regression model, the ucOC level after exercise was associated with insulin sensitivity (GIR and M-value) after exercise ($\beta = 0.66$, $p = 0.005$ and $\beta = 0.39$, $p = 0.02$, respectively) as were BMI and age ($p < 0.05$) but not aerobic fitness. Importantly, baseline ucOC level was also associated with after-exercise insulin sensitivity (GIR and M-value $\beta = 0.57$, $p = 0.036$ and $\beta = 0.37$, $p = 0.044$, respectively) as were age and BMI. The percentage change in ucOC levels from before to after exercise was not associated with the percentage change in whole-body insulin sensitivity after exercise. BMI, age, and aerobic fitness were also not associated with the change in insulin sensitivity from before to after exercise.

UcOC levels after exercise were associated with the change in p-AKT after exercise and insulin ($\beta = 0.69$, $p = 0.03$).

Discussion

We report that acute high-intensity exercise increases ucOC levels and improves insulin sensitivity after exercise. UcOC was associated with insulin sensitivity at rest and after exercise. Insulin sensitivity, at rest and after acute exercise, is associated with circulating levels of ucOC in obese men.

In mice, the skeleton plays a role in glucose metabolism via a mechanism involving ucOC.[5] Data from humans are supportive but are based on cross-sectional studies.[8,12] Because acute exercise is known to increase whole-body insulin sensitivity and glycemic control, it can be used as a tool to examine the contribution of the skeleton to the insulin-sensitizing effect of exercise. We have previously reported that acute aerobic exercise increased both tOC and ucOC in obese men, but only the increase in ucOC was related to an improvement in glycemic control after exercise.[13] Insulin sensitivity and insulin signaling proteins were not measured in the previous study.

It is not clear why tOC did not change in the current study, as previously we have reported that tOC increases with aerobic exercise.[13] It is possible that the different result is related to the exercise intensity or duration. In the current study, participants exercised for 30 minutes at ~95% of HR_{peak} compared with 45 minutes at 75% of HR_{peak} in the previous study. Other studies also reported no change in tOC after aerobic exercise.[22–25] Nevertheless, insulin sensitivity improved in the absence of a change in tOC, suggesting that it is the undercarboxylated form of OC, and not the carboxylated OC, that has a metabolic role in humans, as it does in mice.

There is increasing evidence that ucOC is related to glycemic control in humans; however, causality has not been determined. Although association does not imply causation, our results add

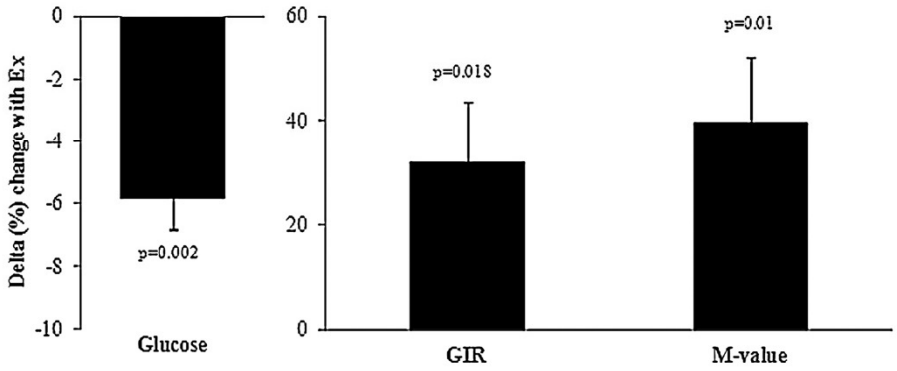

Fig. 3. Acute aerobic exercise reduces blood glucose during the hour after exercise and increases insulin sensitivity 3 hours after exercise. GIR = glucose infusion rate (mL/kg/min); M-value = GIR per unit of serum insulin.

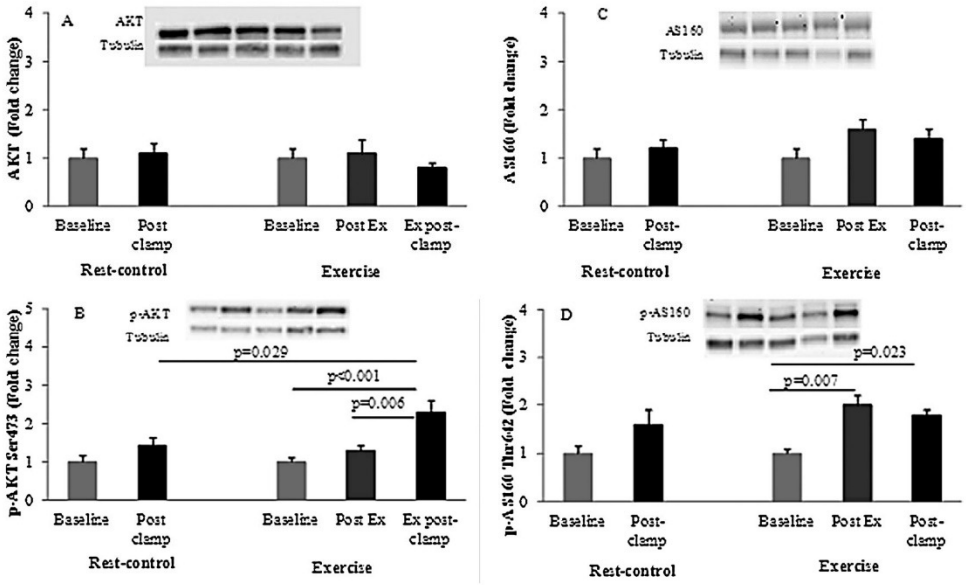

Fig. 4. Acute high-intensity aerobic exercise and insulin infusion have no significant effect on total AKT (*A*) or total AS160 (*C*), but it increases phosphorylated AKT (*B*) and phosphorylated AS160 (*D*).

new information about a possible connection between ucOC and insulin sensitivity in humans. Serum ucOC at baseline was associated with insulin sensitivity and p-AKT at rest and the change in insulin sensitivity after exercise (unadjusted and adjusted for insulin). UcOC level after exercise was also associated with insulin sensitivity after exercise, although the change in ucOC was not associated with the change in insulin sensitivity after exercise. The reason for these conflicting results is not clear. It is possible that those with higher baseline ucOC levels have higher insulin sensitivity at rest and after exercise independent of the relative change in ucOC after exercise. A confirmation for this hypothesis is the moderate, but significant, increase in ucOC after exercise compared with a large change in insulin sensitivity. Hence, the results of the current study suggest that individuals with higher basal ucOC are more likely to have higher insulin sensitivity at rest and after exercise, independent of the change in ucOC after exercise.

Currently, it is unknown whether ucOC has a direct or indirect effect in the insulin-sensing effects of exercise. An indirect pathway by which exercise may improve glycemic control via increases in ucOC is the ucOC effect on islet cells[6] and/or increase in insulin sensitivity[8] that, in turn, may increase glucose uptake by skeletal muscle. A direct effect of ucOC on skeletal muscle is also plausible, although the ucOC receptor in skeletal muscle has yet to be identified. The G protein-coupled receptor 6A (GPCR6A) may be an ucOC receptor in skeletal muscle because it is the ucOC receptor in the Leydig cells of the testes, as well as in pancreatic beta-cells and perhaps in adipocytes and in the liver.[26–28] The GPCR6A receptor has been identified in skeletal muscle and is implicated as a modulator of glucose metabolism.[29] GPCR6A$^{-/-}$ mice have glucose intolerance.[30] Further studies are needed to explore these mechanisms, as well as whether the GPCR6A is in fact the ucOC receptor in skeletal muscle.

The current study reports that exercise increases p-AKT and p-AS160. This is important because both proteins are downstream proteins in the PI3-K pathway regulating GLUT-4 translocation that in turn may increase the muscle capacity to take up more glucose.[14,15] The current study results indicate that the serum ucOC level after exercise was associated with the change in p-AKT after exercise and insulin. It is possible that the pathway by which ucOC increases insulin sensitivity, directly or indirectly, is by activating p-AKT and p-AS160. All together, in humans, similar to mice, ucOC may be partly involved in insulin sensitivity.

A potential limitation of the study is the small sample size. Yet, this sample size was used previously in invasive study that includes several muscle biopsies and two euglycemic-hyperinsulinemic clamps, across two sessions, for each participant.[31,32] The study was powered to detect changes in ucOC, as well as in insulin sensitivity. In addition, we did not assess vitamin K intake, which may affect the carboxylation of OC. However, we have no reason to suspect that this may affect the results of the current study because the ucOC levels at baseline between the two sessions were similar.

In conclusion, acute high-intensity exercise increases ucOC levels and improves insulin sensitivity after exercise. UcOC was associated with insulin sensitivity at rest and after exercise. Insulin sensitivity, at rest and after acute exercise, is associated with circulating levels of ucOC in obese men. Whether ucOC has a direct effect on skeletal muscle insulin sensitivity after exercise is yet to be determined.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: IL, GJ, NS, GM, PE, DH, MA, and ES contributed to design and interpretation. IL, NS, LP, FS, and MA contributed in obtaining the ethical approval, data acquisition, and analysis. IL drafted the initial manuscript, and the remaining authors critically revised the manuscript. All authors approved the final version of the manuscript.

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Acute Low-Volume High-Intensity Interval Exercise and Continuous Moderate-Intensity Exercise Elicit a Similar Improvement in 24-h Glycemic Control in Overweight and Obese Adults

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Background: Acute exercise reduces postprandial oxidative stress and glycemia; however, the effects of exercise intensity are unclear. We investigated the effect of acute low-volume high-intensity interval-exercise (LV-HIIE) and continuous moderate-intensity exercise (CMIE) on glycemic control and oxidative stress in overweight and obese, inactive adults.

Methods: Twenty-seven adults were randomly allocated to perform a single session of LV-HIIE (9 females, 5 males; age: 30 ± 1 years; BMI: 29 ± 1 kg·m^{-2}; mean ± SEM) or CMIE (8 females, 5 males; age: 30 ± 2.0; BMI: 30 ± 2.0) 1 h after consumption of a standard breakfast. Plasma redox status, glucose and insulin were measured. Continuous glucose monitoring (CGM) was conducted during the 24-h period before (rest day) and after exercise (exercise day).

Results: Plasma thiobarbituric acid reactive substances (TBARS; 29 ±13%, $p < 0.01$; mean percent change ±90% confidence limit), hydrogen peroxide (44 ± 16%, $p < 0.01$), catalase activity (50 ± 16%, $p < 0.01$), and superoxide dismutase activity (21 ± 6%, $p < 0.01$) significantly increased 1 h after breakfast (prior to exercise) compared to baseline. Exercise significantly decreased postprandial glycaemia in whole blood (−6 ± 5%, $p < 0.01$), irrespective of the exercise protocol. Only CMIE significantly decreased postprandial TBARS (CMIE: −33 ± 8%, $p < 0.01$; LV-HIIE: 11 ± 22%, $p = 0.34$) and hydrogen peroxide (CMIE: −25 ± 15%, $p = 0.04$; LV-HIIE: 7 ± 26%; $p = 0.37$). Acute exercise provided a similar significant improvement in 24-h average glucose levels (−5 ± 2%, $p < 0.01$), hyperglycemic excursions (−37 ± 60%, $p < 0.01$),

peak glucose concentrations (−8 ± 4%, $p < 0.01$), and the 2-h postprandial glucose response to dinner (−9 ± 4%, $p < 0.01$), irrespective of the exercise protocol.

Conclusion: Despite elevated postprandial oxidative stress compared to CMIE, LV-HIIE is an equally effective exercise mode for improving 24-h glycemic control in overweight and obese adults.

Keywords: HIIT, continuous glucose monitoring, PCOS, redox, postprandial, oxidative stress

INTRODUCTION

Physical inactivity and obesity are major risk factors for impaired glycemic control, insulin resistance and type 2 diabetes (Valko et al., 2007; Fisher-Wellman et al., 2009). Compared to continuous moderate-intensity exercise (CMIE), high-intensity interval exercise (HIIE) has been shown to elicit comparable and/or greater improvements in glycemic control (Gibala et al., 2012; Liubaoerjijin et al., 2016). Notably, an improvement in glycemic control can be seen even after a single bout of exercise (Gillen et al., 2012; van Dijk et al., 2012; Little et al., 2014). However, current laboratory based techniques used for assessing glycemic control, such as the oral glucose tolerance test and the homeostatic model assessment of insulin resistance (HOMA-IR), may not always reflect functional improvements in glycemic control under free-living conditions (Mikus et al., 2012). Continuous glucose monitoring (CGM) is a reliable and valid method for measuring 24-h glycemic status, glycemic variability, and postprandial responses to meals under free-living conditions (Tubiana-Rufi et al., 2007; Mikus et al., 2012). A single bout of HIIE can improve 24-h glycemic control in obese individuals and patients with type 2 diabetes (Gillen et al., 2012; Little et al., 2014). However, only one study has compared the acute effects of HIIE to CMIE when matched for total workload (Little et al., 2014). Consequently, whether shorter duration, lower-volume HIIE (LV-HIIE) provides similar, or greater benefits in 24-h post-exercise glycemic control compared to the currently recommended exercise mode of CMIE is unknown.

Oxidation-reduction (redox) status is reported to mediate glycemic control in both healthy individuals and those with diabetes (Wright et al., 2006; Tiganis, 2011). Oxidative stress occurs as a result of a redox imbalance in favor of excess reactive oxygen species (ROS). This imbalance can result in oxidative modification to DNA, lipids and proteins, playing both a pathological and physiological role in metabolic health (Valko et al., 2007). Chronic systemic oxidative stress is associated with obesity and physical inactivity, and is linked to the development of insulin resistance and type 2 diabetes (Valko et al., 2007). Paradoxically, acute exercise also induces a transient state of elevated oxidative stress (Fisher-Wellman and Bloomer, 2009), yet improves insulin sensitivity and glycemic control (Gillen et al., 2012; van Dijk et al., 2012; Little et al., 2014). While exercise-induced oxidative stress is deemed beneficial and a necessary requirement for optimal tissue functioning and adaptation to physiological stress (Radak et al., 2013), the effects of exercise-intensity on redox status remain unclear.

Elevated basal and/or postprandial hyperglycemia elicited through excess nutrient intake, physical inactivity, and/or insulin resistance, is reported to increase systemic oxidative stress through mitochondrial membrane electron leak and the formation of advanced glycation end products (AGEs) (Wright et al., 2006; Fisher-Wellman and Neufer, 2012). Postprandial oxidative stress can last for up to 4 h after meal consumption and occurs to a greater extent with larger meals that are higher in lipid content (Tucker et al., 2008; Bloomer et al., 2010; Fisher-Wellman and Bloomer, 2010; Fisher-Wellman and Neufer, 2012; Canale et al., 2014). In contrast to exercise-induced oxidative stress, excess postprandial systemic oxidative stress contributes to metabolic health complications associated with insulin resistance and type 2 diabetes (Wright et al., 2006; Tucker et al., 2008; Fisher-Wellman et al., 2009; Fisher-Wellman and Neufer, 2012). A single session of low to moderate-intensity exercise in healthy males attenuates the postprandial oxidative stress response to a meal ingested 2 h before (Mc Clean et al., 2007) and 24 h after exercise (Takahashi et al., 2015). Furthermore, high-intensity exercise which elicits greater oxidative stress and antioxidant activity compared to low to moderate intensity exercise (Schneider et al., 2005; Fisher-Wellman and Bloomer, 2009; Parker et al., 2014), may also attenuate postprandial oxidative stress (Tyldum et al., 2009; Gabriel et al., 2012). Considering the impact of HIIE on postprandial oxidative stress are equivocal (Canale et al., 2014), and overweight and inactive population's exhibit greater basal and postprandial oxidative stress than healthy controls (Tucker et al., 2008; Fisher-Wellman et al., 2009), further research is warranted.

The aim of this study was to test the hypotheses that LV-HIIE would improve 24-h glycemic control and postprandial redox status in overweight and obese males and females to a greater extent than CMIE.

MATERIALS AND METHODS

Participants

Twenty-seven physically inactive males and females, who were on average overweight (BMI range: 21.4–45.0 $kg \cdot m^{-2}$; 23 out of 27 participants had a BMI >25), volunteered to participate in the study. Participant characteristics are reported in **Table 1**. Females diagnosed with polycystic ovary syndrome (PCOS) were included in the study as they have an intrinsic insulin resistance and are at a 4-fold greater risk of developing type 2 diabetes (Stepto et al., 2013; Cassar et al., 2016). PCOS diagnosis was self-reported, and supported by personal medical records that adhered to the Rotterdam criteria (Fauser et al., 2004).

TABLE 1 | Descriptive characteristics of participants in the LV-HIIE and CMIE protocol group.

	LV-HIIE	CMIE	*p*-value
Participants	14	13	
Males	5	5	
Females	9	8	
Females with PCOS	6	5	
Age (years)	30 ± 1	30. ± 2	0.96
Height (cm)	169.5 ± 2.7	166.4 ± 2.3	0.40
Weight	84.1 ± 5.1	83.3 ± 5.6	0.92
BMI ($kg{\cdot}m^{-2}$)	29.2 ± 1.4	30.0 ± 1.8	0.70
BMI >25 $kg{\cdot}m^{-2}$	4 males 8 females	4 males 7 females	
Waist to hip ratio	0.82 ± 0.02	0.84 ± 0.03	0.85
Systolic blood pressure (mm Hg)	122 ± 3	117 ± 3	0.20
Diastolic blood pressure (mm Hg)	85 ± 3	78 ± 3	0.07
W_{max} (Watts)	175 ± 19	170 ± 14	0.82
Max heart rate (BPM)	186 ± 3	180 ± 4	0.22
VO_{2max} ($ml{\cdot}kg^{-1}{\cdot}min^{-1}$)	28.7 ± 2.2	28.8 ± 1.9	0.98
Total exercise session work (kJ)	147 ± 13	191 ± 15*	0.04
Total exercise session duration (min)	24	38*	<0.01
HOMA2-IR	1.4 ± 0.1	1.4 ± 0.2	0.78
Fasting glucose (mmol/l)	4.5 ± 0.1	5.0 ± 0.3	0.12
Fasting insulin (pmol/l)	88 ± 7	84 ± 12	0.78

*Values are mean ± SEM. *p < 0.05 compared to LV-HIIE. LV-HIIE, low-volume high-intensity interval exercise; CMIE, continuous moderate-intensity exercise; PCOS, polycystic ovary syndrome; HOMA2-IR, homeostatic model assessment of insulin resistance version 2. BMI: body mass index.*

Participants were sedentary and had not participated in any regular moderate to high levels of physical activity within the past 3 months. Exclusion for participation included medications known to affect insulin secretion and/or insulin sensitivity; musculoskeletal or other conditions that prevent daily activity; and symptomatic or uncontrolled metabolic or cardiovascular disease. Women with PCOS taking medication (e.g., metformin) were included if medication was stable (>3 months) and were asked to withdraw medication 48 h prior to, and throughout the experimental phase of the study. Females were tested in the early follicular phase of the menstrual cycle. Verbal and written explanations about the study were provided prior to obtaining written informed consent. This study was approved by the Victoria University Human Research Ethics Committee and carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (World Medical, 2013).

Study Design

Participants were instructed to abstain from physical activity, alcohol and caffeine consumption (48 h) prior to, and throughout, the 4 consecutive days of the experimental period. In brief, day one involved insertion of the CGM and participant familiarization; Day two was a rest day with dietary control; Day three was an exercise day (LV-HIIE or CMIE) with dietary control; Day four involved the removal of the CGM (**Figure 1**).

Screening and Preliminary Testing

Participants were pre-screened via a medical history and risk assessment questionnaire. Eligible participants underwent body composition analysis, and a graded exercise test (GXT) on a cycle ergometer (Lode Excalibur Sport) to measure aerobic capacity (VO_{2max}) and maximal power output (W_{max}). Expired gases were collected and analyzed via a metabolic system (Moxus Modular VO2 System). The GXT protocol consisted of 3 min cycling (60 RPM) at 50 W, increasing by 25 W every 3 min for the first three stages, and then increasing every 1 min thereafter. Participants cycled until they were unable to maintain 50 revolutions per min. The maximum wattage obtained (W_{max}) during the exercise test was used to calculate the workload for the LV-HIIE or CMIE in the main experiment. Heart rate (HR) was recorded using a 12-lead electrocardiograph system along with the 6–20 Borg scale rating of perceived exertion (RPE). One to three weeks after completing the pre-screening session participants were randomized into either the LV-HIIE or CMIE exercise interventions stratified by sex and BMI.

EXPERIMENTAL PHASE

Experimental Day 1

Participants reported to the laboratory for fitting of the CGM (Guardian® Real-Time, Medtronic, USA) and detailed instructions of its use (Tubiana-Rufi et al., 2007). Calibration of the CGM was performed three times per day (morning, lunchtime, and evening), and performed at least 2 h after the participants last meal. Participants were instructed to consume the food provided and refrain from any physical activity not prescribed by the researchers. Prior to leaving the laboratory, participants were given a standardized meal (dinner) to take home and consume at their normal meal time.

Experimental Day 2

Participants reported to the laboratory in the morning after an overnight fast and consumed a standardized breakfast. Immediately following breakfast participants rested in the laboratory for a total of 3 h. Blood samples were taken at baseline (prior to breakfast) and 3 h after breakfast. Participants were provided standardized meals for lunch, dinner and snacks to take home and eat at their preferred meal times, and were instructed to detail all food consumption and physical activity in the provided log books.

Experimental Day 3

Participants again reported to the laboratory in the morning after an overnight fast. As per the previous rest day, participants consumed the standardized breakfast. One hour later, participants performed a single session of either the LV-HIIE or CMIE protocol. The participants then rested in the laboratory for 90 min. The standardized meals were again

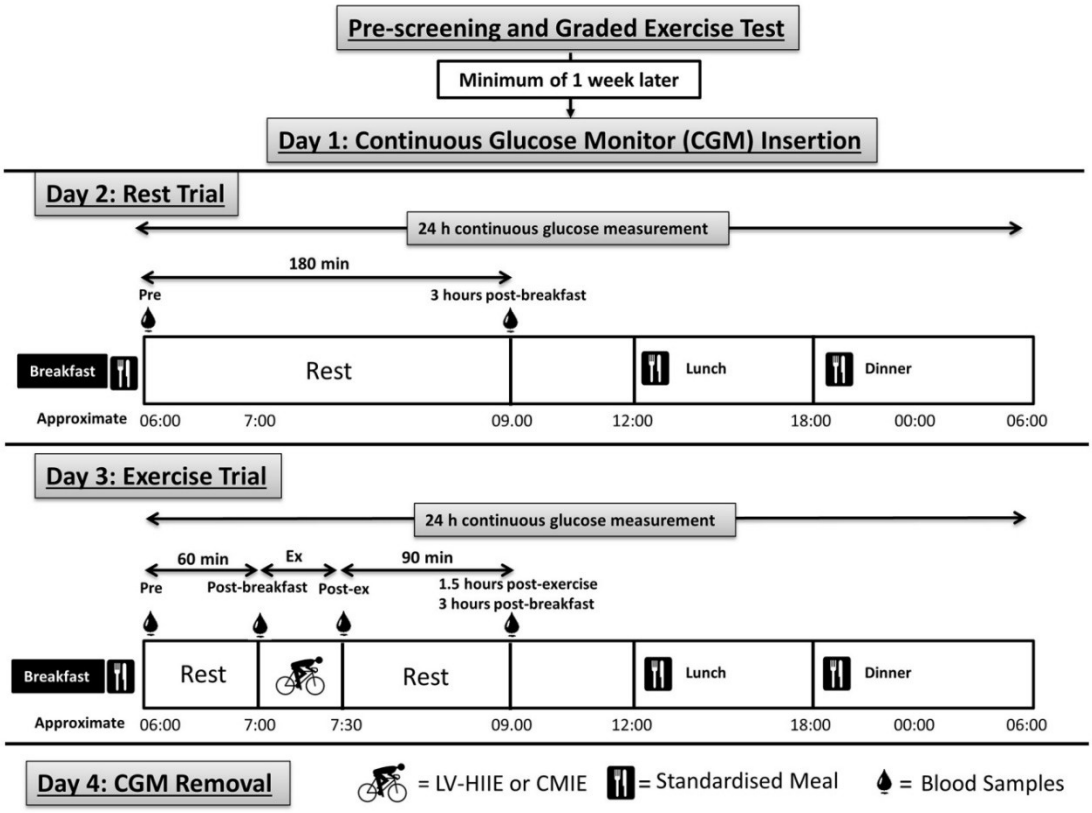

FIGURE 1 | Detailed schematic of the research methodology and procedures. Participants were pre-screened and randomized into either LV-HIIE or CMIE exercise groups. Participants than underwent 4 consecutive days of testing. Day 1 consisted of CGM insertion and instruction of its appropriate use. Day 2 consisted of a rest non-exercise day. Day 3 was identical to day 2 with the addition of a single session of either LV-HIIE or CMIE performed 1 h after breakfast consumption. Day 4 consisted of removal of the CGM. Participants were provided a standard breakfast, lunch, dinner and snacks, to eat throughout the 4-day testing period. Meals were consumed at the same time of day over the testing period. Venous blood samples were taken at the time points indicated in the figure. CMIE, Continuous moderate-intensity exercise; LV-HIIE, low-volume high-intensity interval exercise; CGM, continuous glucose monitor.

provided including the final day's breakfast (day 4), to be consumed at the same time of day as per the previous 2 days. Blood samples were taken at baseline, pre-exercise (1 h after breakfast), and immediately after exercise, 5, 15, 60, and 90 min after exercise (∼3 h after breakfast).

Experimental Day 4

Participants arrived in the laboratory ∼3 h after consumption of their standard breakfast and the CGM was removed.

Exercise Protocols

The LV-HIIE protocol consisted of a 5-min warm-up at 50% of the participants W_{max} obtained during the GXT. Following the warm-up, participants performed 8 × 1-min cycling bouts at 100% of W_{max} (175 ± 19 W), interspersed with 1-min active recovery periods cycling at 50 W. A 3-min cool down was then performed at 50% of W_{max}. The total workout session duration was 24 min. The CMIE session consisted of 38 ± 1 min cycling at 50% of the participants W_{max} (79 ± 9 W). Total work performed on the cycle ergometer during the LV-HIIE and CMIE sessions are reported in **Table 1**.

Dietary Control

Daily energy and macronutrient intake for the standardized meals were based on sex, height and weight, and consisted of approximately 55% carbohydrate, 30% fat and 15% protein, adhering with the Australian and New Zealand dietary targets (Council, 2014). With the exception of necessary dietary substitutions (vegetarian, halal etc.) breakfast consisted of Kellogg's® Corn Flakes and Kellogg's® All-Bran®, honey and full cream milk; lunch consisted of canned tuna, tomato, lettuce and carrot roll/s; dinner consisted of sausages, cooked white rice, sweet potato, and mixed frozen vegetables; and

snacks consisted of a muffin, banana, and yogurt. To ensure consistency throughout the study, participants were instructed to eat breakfast, lunch and dinner at the same time of day over the 4-day experimental period, and were instructed to log all physical activity (time of day, exercise mode, duration and intensity) and food consumed (time of day, type and quantity of food eaten) in the provided log books.

Blood Sampling

Venous blood was collected from an antecubital vein via an intravenous cannula and collection tube and kept patent with 0.9% sterile saline. Blood was collected in appropriate tubes and immediately centrifuged at 3500 rpm for 15 min at 4°C, the plasma was aliquoted and stored at −80°C until analyzed.

Biochemical Analysis

Whole blood lactate and glucose were analyzed using an automated analysis system (YSI 2300 STAT Plus® Glucose & Lactate Analyzer). Plasma insulin levels were determined in duplicate using radioimmunoassay in accordance with the manufacturer's instructions (HI-14K kit, Millipore). Insulin resistance was estimated using the homeostatic model assessment (version 2) for insulin resistance (HOMA2-IR) using the Oxford Diabetes Trials Unit calculator (https://www.dtu.ox.ac.uk/homacalculator; University of Oxford, UK).

Plasma Redox Status Analysis

Plasma thiobarbituric acid reactive substances (TBARS; Cayman), catalase activity (Cayman), superoxide dismutase activity (SOD; Cayman) and hydrogen peroxide (Amplex UltraRed assay, Molecular Probes) were determined on a spectrophotometer (xMark microplate spectrophotometer, Bio-Rad Laboratories) in duplicate as per the manufacturer's instructions. Intra-assay coefficients of variation were 2, 3, 3, and 2% for TBARS, SOD, Catalase and hydrogen peroxide, respectively. Inter-assay coefficients of variation were 1, 4, 4, and 1%, for TBARS, SOD, catalase and hydrogen peroxide, respectively.

Continuous Glucose Monitor Analysis

Five-minute glucose values recorded by the CGM over the 4-day intervention were exported. Meal-times were cross-checked with participants' diet log books and data were checked for missing values and/or abnormal readings. The 24-h period prior to the onset of exercise (rest day) and the 24-h period immediately after the onset of exercise (exercise day), were used to compare 24-h CGM determined glycemic control. For consistency, missing data points were handled as per previous publications using CGM technology (Little et al., 2014). Briefly, if less than 3 consecutive 5-min periods were missing the average of the glucose value before and after were inserted. If greater than 3 consecutive 5-min periods were missing over the 24-h period of comparison, then both the rest and exercise days were adjusted to omit these values. Continuous glucose monitoring data was corrupted for 1 participant in the LV-HIIE, and was excluded from the CGM comparisons.

Average glucose values, peak glucose concentration, total area under the curve (AUC) and incremental AUC (iAUC), were calculated for the 24-h rest day and exercise day period, and the 2-h postprandial period following consumption of breakfast, lunch and dinner. The 24-h glycemic variability measurements of the standard deviation (SD) of the mean glycaemia, the mean amplitude of glycemic excursions (MAGE), and the percentage coefficient of variation (% CV), were calculated using the GlyCulator windows software package as previously described (Czerwoniuk et al., 2011). The percentage of time spent with hyperglycemia (above 7 mmol/l) during the 24-h time period was also determined.

Statistical Analysis

Data were checked for normality and analyzed using Predictive Analytics Software (PASW v20, SPSS Inc.). Comparisons of means for the CGM data were examined using a two-factor repeated measures analysis of variance (ANOVA) with trial day (rest or exercise day) as the within-subject factor and group (LV-HIIE or CMIE) as a between-subjects factor. Comparison of multiple means for biochemical analysis, heart rate and RPE, on the exercise day were analyzed using a two-factor repeated measures ANOVA with time as the within-subjects factor and group (LV-HIIE and CMIE) as the between-subjects factor. A three-factor repeated measures ANOVA was conducted to investigate the effect of exercise on postprandial oxidative stress with time (baseline and 3 h postprandial) and day (rest day and exercise day) as within-subject factors and group (LV-HIIE and CMIE) as the between-subjects factor. Significant interaction and main effects were explored using Fisher's LSD *post-hoc* analysis test. All data are reported as mean ± standard error of mean (SEM) and all statistical analysis were conducted at the 95% level of significance ($p \leq 0.05$). Trends were reported when p-values were greater than 0.05 and less than 0.1.

RESULTS

Measures of fasting glucose, fasting insulin, and HOMA2-IR were not statistically different between participants with and without PCOS, or between PCOS participants in the LV-HIIE and CMIE groups ($p > 0.05$; data not shown), as such data were pooled together for analysis. Baseline measurements for CGM and biochemical data, and physiological data measured in the GXT, were not significantly different ($p > 0.05$) between LV-HIIE and CMIE groups (**Table 1**).

Physiological Response to Exercise

HR and RPE were significantly higher during LV-HIIE compared to CMIE (**Figure 2**). Total work (kJ) and exercise duration (minutes) in the CMIE group were significantly greater than LV-HIIE (**Table 1**).

Continuous Glucose Monitor Data

Average glucose concentration, glucose AUC, peak glucose concentration, and the percentage of the day spent with glucose values above 7 mmol/l were significantly lower during the 24-h period after exercise compared to the rest day (all $p < 0.05$) with

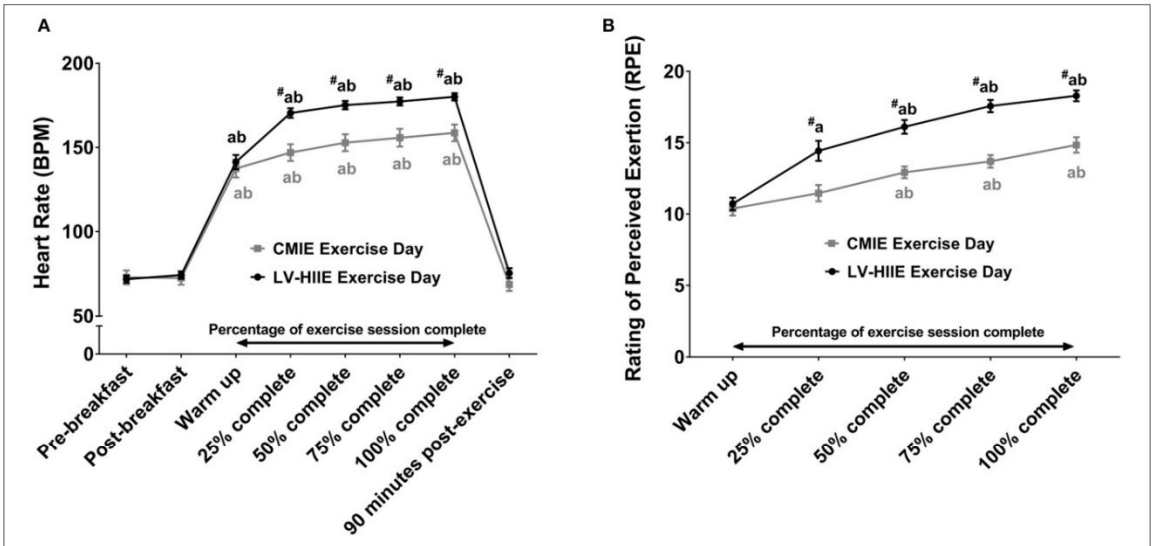

FIGURE 2 | Rating of perceived exertion and heart rate response to LV-HIIE and CMIE. Heart rate **(A)** and rating of perceived exertion **(B)** during exercise for both LV-HIIE and CMIE. $^{a}p < 0.05$ compared to pre-breakfast or warm up; $^{b}p < 0.05$ compared to 1-h post-breakfast or repetition 2 (25% of CMIE); $^{\#}p < 0.05$ compared to the corresponding time point in the CMIE group. CMIE, continuous moderate intensity exercise; LV-HIIE, low-volume high-intensity interval exercise. Values are means ± SEM.

no differences between LV-HIIE and CMIE (all $p > 0.05$; **Table 2**). Exercise significantly decreased dinner 2-h average glucose concentration, peak glucose concentration, AUC and iAUC (all $p < 0.05$; **Table 2**). Exercise significantly decreased breakfast total and iAUC (both $p < 0.05$), whereas a significant interaction effect ($p = 0.04$) and subsequent *post-hoc* analysis indicated that only CMIE decreased breakfast 2-h average glucose concentration ($p < 0.01$; **Table 2**). The glycemic variability measurements of MAGE, SD, and % CV were not significantly different between trial days or groups (all $p > 0.05$; **Table 2**). Representative graphs of the mean difference between the rest day and exercise day over the 24-h period, and the CGM postprandial breakfast response, are reported in **Figure 3**.

Exercise Day Biochemical Analysis

Blood glucose, plasma insulin, hydrogen peroxide, TBARS, catalase activity, and SOD activity were not significantly different between groups at baseline (all $p > 0.05$). Significant interaction effects were detected for plasma insulin ($p = 0.01$), glucose ($p = 0.01$), lactate ($p < 0.01$), hydrogen peroxide ($p = 0.05$), and TBARS ($p < 0.01$). *Post-hoc* analysis revealed that plasma insulin was significantly higher 1 h after breakfast compared to baseline for both the LV-HIIE and CMIE group (**Figure 4**). Immediately, and 1.5 h after CMIE, insulin levels returned to baseline. This was not evident after LV-HIIE where insulin levels remained significantly elevated. Blood glucose levels were significantly lower than baseline immediately after exercise with CMIE, and significantly lower than baseline 1.5 h after exercise after both CMIE and LV-HIIE (**Figure 4**). Blood lactate was significantly higher at all-time points after LV-HIIE compared to CMIE (**Figure 4**).

Plasma hydrogen peroxide, TBARS, catalase activity, and SOD activity were significantly higher 1 h after breakfast (**Figure 5**). SOD activity and catalase activity remained significantly elevated during the recovery period after both CMIE and LV-HIIE. On the other hand, *post-hoc* analysis of hydrogen peroxide and TBARS remained significantly elevated during the recovery period after LV-HIIE only (**Figure 5**).

The Effect of Exercise on 3-h Postprandial Oxidative Stress

A significant interaction (group∗time∗day) effect ($p = 0.03$) was detected for hydrogen peroxide. *Post-hoc* analysis indicated that compared to baseline hydrogen peroxide was significantly elevated 3 h after breakfast on the rest day with both CMIE and LV-HIIE groups ($p < 0.05$; **Table 3**). Furthermore, 3-h postprandial hydrogen peroxide was elevated to a greater extent ($p < 0.05$) on the exercise day compared to the rest day with LV-HIIE but not CMIE (**Table 3**). There was a trend toward significance ($p = 0.06$) for an interaction effect (group*time*day) for TBARS (**Table 3**). *Post-hoc* analysis indicated that compared to the rest day there was a tendency for greater TBARS at 3 h postprandial compared to the exercise day with LV-HIIE ($p = 0.08$) and lower TBARS on the exercise day with CMIE ($p = 0.07$). Furthermore, TBARS was significantly greater on the exercise day at 3 h postprandial with LV-HIIE compared to CMIE ($p < 0.05$; **Table 3**). A main time effect ($p < 0.01$)

TABLE 2 | Analysis of continuous glucose monitoring measurements during the rest and exercise day for both LV-HIIE and CMIE sessions.

Variable	LV-HIIE		CMIE	
	Rest day	Exercise day	Rest day	Exercise day
24 h MEASUREMENT (mmol/l)				
Average blood glucose	4.7 ± 0.1	4.5 ± 0.1*	5.3 ± 0.3	5.1 ± 0.3*
Total AUC	6438 ± 192	6077 ± 214*	7174 ± 506	6847 ± 475*
Peak glucose concentration	7.2 ± 0.3	6.5 ± 0.3*	7.9 ± 0.6	7.2 ± 2.0*
2 h PPG (mmol/l)				
Breakfast	5.1 ± 0.2	5.3 ± 0.3	6.1 ± 0.5	5.4 ± 0.4*
Breakfast (1st h)	5.4 ± 0.2	5.4 ± 0.2	6.2 ± 0.4	5.8 ± 0.4
Breakfast (2nd h)	4.9 ± 0.2	4.5 ± 0.2*	5.9 ± 0.6	5.0 ± 0.4*
Lunch	5.0 ± 0.2	5.1 ± 0.1	5.9 ± 0.3	5.7 ± 0.3
Dinner	5.2 ± 0.3	4.6 ± 0.2*	6.2 ± 0.4	5.7 ± 0.3*
2 h PPP (mmol/l)				
Breakfast	6.1 ± 0.2	6.3 ± 0.3	7.4 ± 0.7	6.8 ± 0.6
Lunch	5.7 ± 0.2	5.9 ± 0.3	7.1 ± 0.4	6.8 ± 0.5
Dinner	5.9 ± 0.4	5.2 ± 0.3*	7.1 ± 0.5	6.5 ± 0.4*
2 h iPPP (mmol/l)				
Breakfast	1.6 ± 0.2	1.7 ± 0.3	2.4 ± 0.4	1.9 ± 0.3
Lunch	1.5 ± 0.2	1.6 ± 0.3	2.5 ± 0.3	2.0 ± 0.4
Dinner	1.5 ± 0.3	1.1 ± 0.2*	2.1 ± 0.3	1.8 ± 0.3*
2 h AUC (mmol/l·2 h)				
Breakfast	617 ± 25	609 ± 29*	730 ± 63	655 ± 49*
Lunch	601 ± 25	610 ± 16	711 ± 36	709 ± 32
Dinner	629 ± 34	569 ± 23*	748 ± 53	687 ± 41*
2 h iAUC (mmol/l·2 h)				
Breakfast	88.6 ± 11.9	80.5 ± 18.6*	105.0 ± 13.7	71.3 ± 14.0*
Lunch	107.7 ± 16.2	93.3 ± 11.7	173.1 ± 20.8	153 ± 27.7
Dinner	107.2 ± 18.0	75.9 ± 20.4*	154.3 ± 30	129.4 ± 26.9*
GLYCEMIC VARIABILITY				
MAGE	2.2 ± 0.2	2.2 ± 0.2	2.6 ± 0.3	2.2 ± 0.3
SD	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
%CV	15.1 ± 1.2	15.7 ± 1.6	15.58 ± 1.3	14.58 ± 1.7
% of 24 h day at >7 mmol/l[δ]	2.7 ± 0.9	0.2 ± 0.2*	11.4 ± 6.3	8.5 ± 5.3*

*Values are mean ± SEM (n = 13 in both LV-HIIE and CMIE groups for CGM data). *p < 0.05 are significantly different to the rest day. δ-7 out of 13 participants in the LV-HIIE group and 10 out of 13 in the CMIE group exhibited glucose values higher than 7 mmol/L. LV-HIIE, low-volume high-intensity interval exercise; CMIE, continuous moderate-intensity exercise; MAGE, 24-h mean amplitude of glycemic excursion; SD, 24-h glycemic standard deviation. %CV, 24-h glucose percentage coefficient of variation; PPG, postprandial glucose; PPP, peak postprandial glucose; iPPP, incremental peak postprandial glucose; AUC, area under the glucose curve; iAUC, incremental area under the glucose curve.*

indicated significantly greater catalase activity at 3 h postprandial compared to baseline irrespective of exercise group or day (**Table 3**). A significant interaction (group*time*day; $p = 0.043$) effect was detected for SOD activity. *Post-hoc* analysis indicated significantly greater SOD activity at 3 h postprandial compared to baseline with both CMIE and LV-HIIE groups on the rest day, and with LV-HIIE on the exercise day (all $p < 0.05$). Compared to the rest day, there was a trend ($p = 0.051$) for decreased SOD activity at 3 h postprandial on the exercise day with CMIE.

Comparison of the change (3-h postprandial value minus the baseline value) between the rest day and exercise days indicated that LV-HIIE elicited a greater increase in hydrogen peroxide ($p = 0.03$), TBARS ($p = 0.06$), and SOD activity ($p = 0.04$) on the exercise day compared to CMIE.

DISCUSSION

We report the novel finding that LV-HIIE performed 1 h after consumption of a standard breakfast elicits a greater postprandial oxidative stress response compared to CMIE. Yet, over the 24-h post-exercise period LV-HIIE improves glycemic control to a similar extent as CMIE in overweight and obese adults. LV-HIIE consisted of significantly less work and time commitment compared to CMIE and therefore appears to be an effective exercise mode for incorporation into exercise programs designed to improve glycemic control in overweight and obese populations including insulin resistant conditions like PCOS.

Acute Exercise and 24-h Glycemic Control

Previous research indicates that a single bout of endurance exercise improves insulin sensitivity in the hours after exercise (Gillen et al., 2012; van Dijk et al., 2012; Little et al., 2014), however the effects of HIIE on glycemic control in sedentary overweight and obese populations are less clear. Laboratory based methods, such as the hyperinsulinaemic-euglycemic clamp and oral glucose tolerance test, may not reflect physiological insulin and glucose dynamics (Muniyappa et al., 2008) and can underestimate functional improvements in glycemic control (Mikus et al., 2012). We report the novel finding that 24-h post-exercise glycemic control measured under free living conditions is improved with LV-HIIE to a similar extent as CMIE. Manders et al. (2010) previously reported that CMIE (60 min at 35% W_{max}) elicited greater improvements in 24-h glycemic control compared to higher-intensity exercise (30 min at 70% W_{max}) in participants with type 2 diabetes. The discrepancy in findings may be a result of the different populations investigated, participants with type 2 diabetes vs. overweight/obese adults. However, Terada et al. (2016) recently reported greater improvements in 24-h glycemic control and postprandial glycemia after fasted-state HIIE (15 × 1 min at 100% VO_{2peak}; 3-min active recovery periods) compared to work-matched CMIE treadmill exercise (60 min at 55% $VO2_{peak}$) in participants with type 2 diabetes. Furthermore, HIIE cycling (10 × 1 min at 90% W_{peak}; 1 min recovery periods at 15% W_{peak}) in overweight/obese adults elicits greater improvements in 24-h glycemic control compared to work-matched CMIE (30 min at 35% W_{peak}; Little et al., 2014). Combined with the current findings, HIIE is a potent stimulus for improving glycemic control, with potentially greater benefits occurring with HIIE of sufficient volume. In contrast, research exploring sprint-interval exercise reported no improvement in insulin sensitivity at 24 and 72 h post-exercise (Richards et al., 2010; Whyte et al., 2013), although insulin sensitivity was improved 24 h after an extended sprint work-matched to sprint-interval exercise (Whyte et al., 2013). However, these studies did not measure glycemic control during the post-exercise recovery

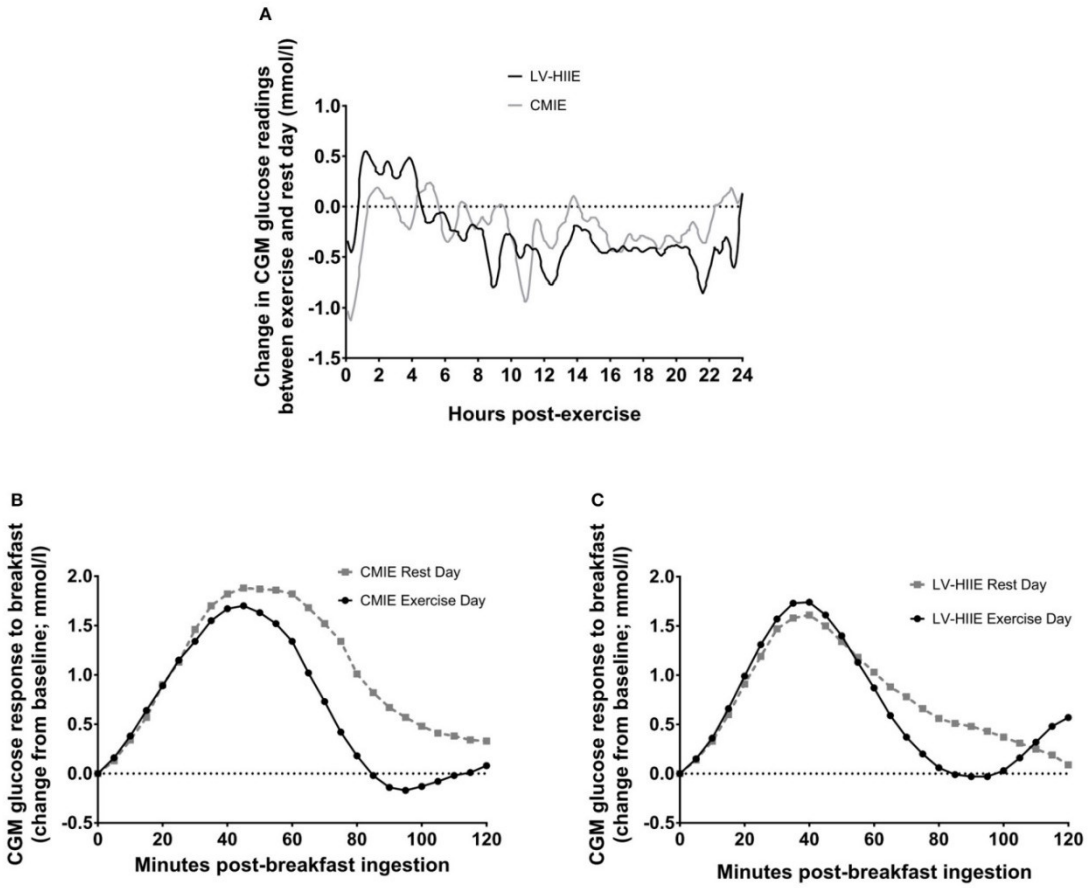

FIGURE 3 | Continuous glucose monitor traces for 24-h glucose and postprandial breakfast. Change in average CGM glucose readings (mmol/l) from rest to exercise day over the 24-h period **(A)**. Average postprandial CGM glucose readings (change from baseline; mmol/l) on the rest day and when CMIE **(B)** or LV-HIIE **(C)** were performed 1 h after breakfast consumption. CMIE, continuous moderate intensity exercise; LV-HIIE, low-volume high-intensity interval exercise; CGM, continuous glucose monitor.

period (Richards et al., 2010; Whyte et al., 2013). We extend previous findings by reporting that LV-HIIE, which consisted of substantially less time commitment and total work than CMIE, elicits similar improvements in 24-h post-exercise glycemic control.

Regulation of glycemic fluctuations and postprandial glycaemia are important for the long-term maintenance of insulin sensitivity and lowered risk of metabolic disease (Wright et al., 2006). Similar to previous reports (Little et al., 2014), we did not detect significant changes in glycemic variability as measured by MAGE, SD, and CV, with either LV-HIIE or CMIE. It is possible that differences were not detected, as CGM readings have been reported to underestimate measures of glycemic variability (Akintola et al., 2015). Furthermore, greater glycemic variability is strongly associated with a history of diabetes, suggesting that glycemic variability may play less of a role in apparently healthy populations who have regular glycemic control (Sartore et al., 2012). Nevertheless, the significant reductions in hyperglycemia, improved postprandial dinner response and improved 24-h average blood glucose and AUC, identify LV-HIIE as a beneficial exercise mode for improving overall glycemic control in sedentary, overweight and obese individuals. Indeed, training programs incorporating LV-HIIE are reported to promote long term improvements in glycemic control (Gibala et al., 2012). We extend these findings by reporting that a single session of LV-HIIE or CMIE can have similar improvements in glycemic control for up to 24 h after exercise has ceased.

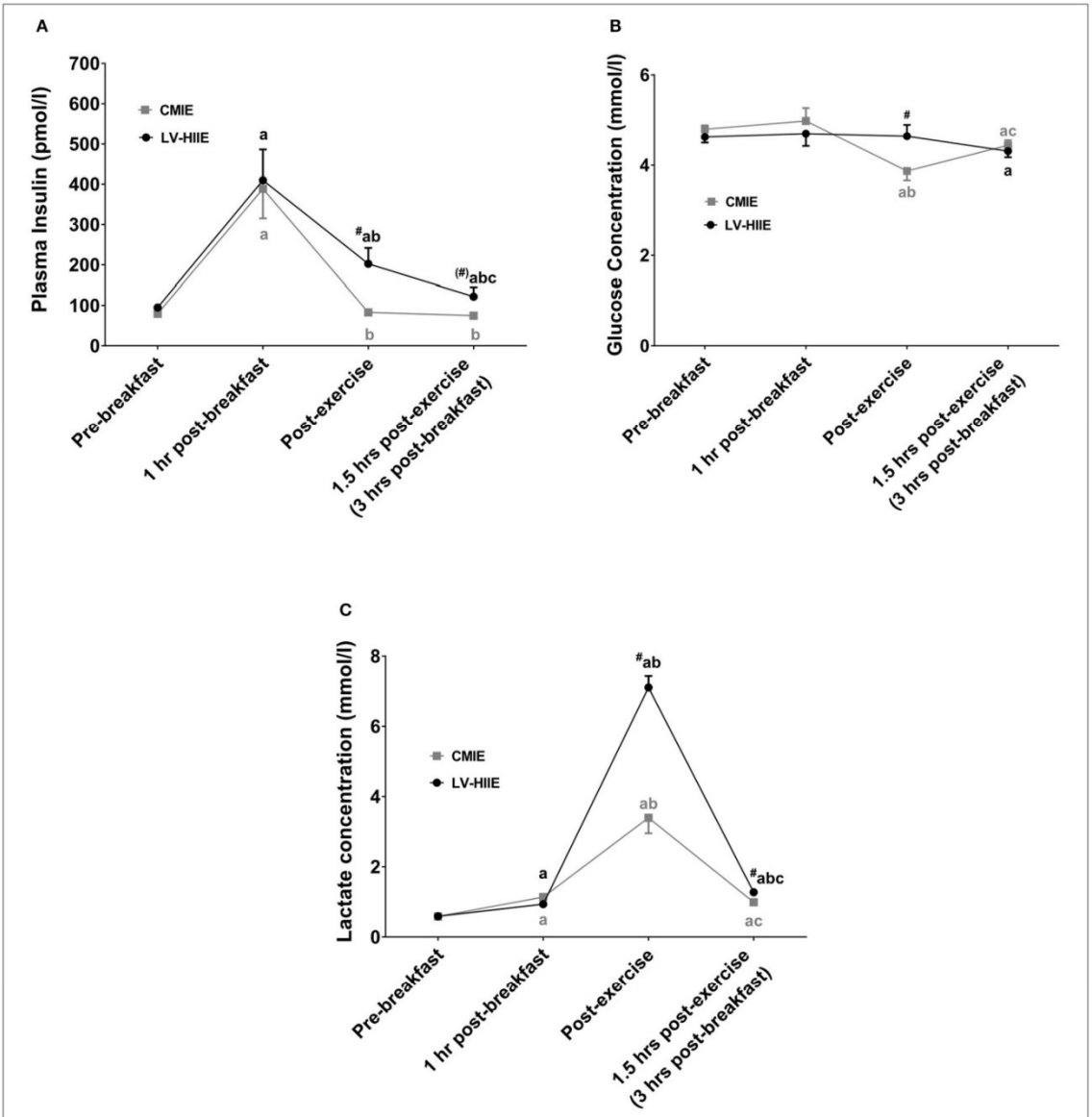

FIGURE 4 | Biochemical blood and plasma response to breakfast and exercise. The postprandial and post-exercise response of plasma insulin **(A)**, blood glucose **(B)**, and blood lactate **(C)**. $^{a}p < 0.05$ compared to baseline; $^{b}p < 0.05$ compared to 1 h post-breakfast; $^{c}p < 0.05$ compared to post-exercise; $^{\#}p < 0.05$ compared to the equivalent time point in the CMIE group. Symbols in parenthesis indicate $p < 0.1$. CMIE, continuous moderate intensity exercise; LV-HIIE, low-volume high-intensity interval exercise. Values are means ± SEM.

Redox Status Response to Exercise Performed 1 h after Breakfast

Excessive oxidative stress leads to the development and progression of numerous pathologies including insulin resistance and type 2 diabetes (Valko et al., 2007; Fisher-Wellman and Neufer, 2012). Increased energy substrate availability following the consumption of a meal, results in elevated ROS production through mitochondrial membrane electron leak and the formation of AGEs (Tucker et al., 2008; Fisher-Wellman and Neufer, 2012). Excess ROS activate stress and mitogen activated

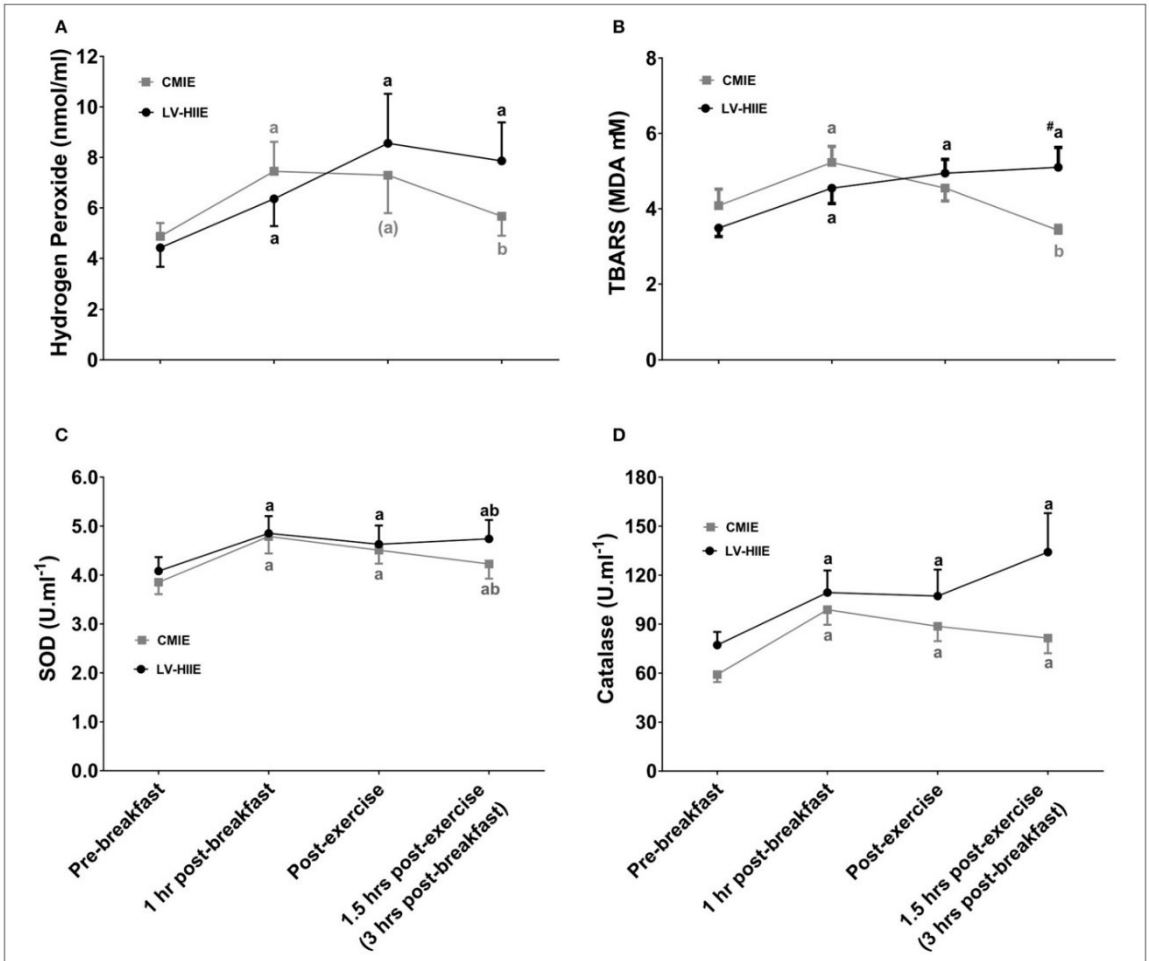

FIGURE 5 | Plasma redox status response to breakfast and exercise. Plasma redox status response to ingestion of a standard breakfast and when either LV-HIIE or CMIE was performed 1 h after breakfast. The postprandial and post-exercise response of plasma hydrogen peroxide **(A)**, TBARS **(B)**, SOD activity **(C)**, and catalase activity **(D)**. $^{a}p < 0.05$ compared to baseline; $^{b}p < 0.05$ compared to 1 h post-breakfast; $^{\#}p < 0.05$ compared to the equivalent time point in the CMIE group. Symbols in parenthesis indicate $p < 0.1$. CMIE, continuous moderate intensity exercise; LV-HIIE, low-volume high-intensity interval exercise; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances. Values are means ± SEM.

protein kinase signaling pathways in insulin sensitive tissues contributing to the development of insulin resistance and type 2 diabetes (Wright et al., 2006; Tiganis, 2011). However, research has also highlighted exercise-induced ROS as a prominent moderator of glycemic control (Ristow et al., 2009; Trewin et al., 2015; Parker et al., 2016).

We report increased postprandial oxidative stress (TBARS and hydrogen peroxide) and antioxidant activity (catalase and SOD activity) 1 h after breakfast. In addition, hydrogen peroxide, catalase and SOD activity remain elevated 3 h after breakfast. Interestingly, only CMIE attenuated this postprandial oxidative stress response as evident by decreased TBARS and hydrogen peroxide 1.5 h after exercise compared to pre-exercise values, and decreased plasma TBARS 1.5 h after exercise compared to the rest day. Taken together with previous work (Canale et al., 2014), our findings suggest that postprandial oxidative stress is attenuated by CMIE, possibly due to improved clearance of plasma glucose. Certainly, others have reported that CMIE (1 h at 60% HR_{max}) performed 2 h after a high fat meal attenuates postprandial oxidative stress in trained males (Mc Clean et al., 2007). In contrast, others have reported that cycling at 65–70% heart rate reserve for 45–60 min did not attenuate postprandial oxidative stress (Melton et al., 2009; Canale et al., 2014). The discrepancy in findings are likely related to the timing of meal

TABLE 3 | The effect of exercise intensity and volume on plasma postprandial oxidative stress.

	Rest Day			Exercise Day			
	Baseline	3 h postprandial	Change on rest day (Cohens d)	Baseline	3 h postprandial	Change on exercise day (Cohens d)	Change between exercise and rest day (Cohens d)
HYDROGEN PEROXIDE (nmol.ml^{-1})							
LV-HIIE	4.5 ± 0.8	5.6 ± 1.0*	1.1 ± 0.5 (0.37)	4.4 ± 0.7	7.9 ± 1.5*†	3.4 ± 1.3 (1.46)	2.3 ± 1.1# (1.44)
CMIE	5.4 ± 0.8	6.8 ± 1.0*	1.4 ± 0.4 (0.46)	4.9 ± 0.5	5.7 ± 0.7	0.8 ± 0.6 (0.34)	−0.6 ± 0.4 (−0.36)
TBARS (MDA μM)							
LV-HIIE	3.2 ± 0.2	4.0 ± 0.3	0.8 ± 0.3 (0.68)	3.5 ± 0.2	5.1 ± 0.5*#†	1.6 ± 0.6 (1.30)	0.8 ± 0.8# (0.52)
CMIE	4.1 ± 0.4	4.6 ± 0.4	0.5 ± 0.5 (0.44)	4.1 ± 0.4	3.4 ± 0.1†	−0.7 ± 0.4 (−0.53)	−1.2 ± 0.5 (−0.77)
CATALASE (U.ml^{-1})							
LV-HIIE	32.6 ± 2.4	42.9 ± 5.9*	10.3 ± 5.1 (1.24)	32.8 ± 3.3	57 ± 9.8*	24.2 ± 8.3 (2.25)	13.9 ± 8.4 (0.73)
CMIE	25.2 ± 1.6	39.2 ± 6.0*	14.0 ± 5.2 (1.69)	25.1 ± 1.9	34.6 ± 3.8*	9.5 ± 3.9 (0.88)	−4.5 ± 6.0 (−0.24)
SOD (U.ml^{-1})							
LV-HIIE	4.1 ± 0.3	4.5 ± 0.3*	0.4 ± 0.1 (0.44)	4.1 ± 0.3	4.7 ± 0.4*	0.7 ± 0.3 (0.69)	0.2 ± 0.2# (0.50)
CMIE	3.9 ± 0.3	4.6 ± 0.3*	0.7 ± 0.1 (0.76)	3.9 ± 0.2	4.2 ± 0.3†	0.4 ± 0.2 (0.39)	−0.3 ± 0.2 (−0.68)

*Values are mean ± SEM. LV-HIIE, low-volume high-intensity interval exercise; CMIE, continuous moderate-intensity exercise; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; *$p < 0.05$ compared to baseline; †$p < 0.05$ compared to rest day; #$p < 0.05$ compared to CMIE; symbols in parenthesis indicate $p < 0.1$.*

ingestion, with improvements in postprandial oxidative stress occurring when exercise is performed in the hours after meal ingestion (Mc Clean et al., 2007), whereas exercising prior to meal ingestion may be less effective (Melton et al., 2009; Canale et al., 2014).

A novel finding was that plasma hydrogen peroxide and TBARS were elevated 1.5 h after LV-HIIE (approximately 3 h after breakfast) compared to the rest day. The mechanisms for the divergent oxidative stress response between LV-HIIE and CMIE are unclear. Oxidative stress is reported to be greater after higher-intensity exercise (Fisher-Wellman and Bloomer, 2009) and likely occurs through pathways independent of postprandial-induced oxidative stress (Fisher-Wellman and Bloomer, 2009; Fisher-Wellman and Neufer, 2012; Radak et al., 2013). Thus, it is likely that elevated oxidative stress after LV-HIIE is a result of increased exercise-induced oxidative stress. Additionally, it is possible that comparatively lower blood glucose after CMIE may allow for decreased mitochondrial electron leak, AGE formation, and subsequent ROS production (Wright et al., 2006; Tucker et al., 2008; Fisher-Wellman and Neufer, 2012).

Exercise-induced oxidative stress and subsequent redox-sensitive protein signaling facilitate many of the health benefits of acute and regular exercise (Ristow et al., 2009; Radak et al., 2013; Trewin et al., 2015; Parker et al., 2016). Furthermore, many of the metabolic health benefits of higher-intensity exercise occur during the delayed exercise recovery period (i.e., the day after exercise), potentially through alterations in redox status (Tyldum et al., 2009; Gabriel et al., 2012). It is possible that increased exercise-induced oxidative stress after LV-HIIE may be beneficial. Further research is required to elucidate the effect of LV-HIIE on redox status during the 24-h post-exercise recovery period.

Glucoregulatory Response to Exercise Performed 1 h after Breakfast

Elevated postprandial glycemia is reported to play a role in the development of insulin resistance and metabolic disease (Wright et al., 2006). Similar to previous research (van Dijk et al., 2013), we demonstrate that a single session of CMIE performed 1 h after breakfast attenuates postprandial glycemia. We extend previous findings by indicating that LV-HIIE also attenuates postprandial glycemia, despite consisting of considerably less total work and time-commitment. CMIE decreased whole blood glucose immediately after exercise compared to baseline and LV-HIIE. This reduction was transient as both LV-HIIE and CMIE elicited a similar decrease in whole blood and CGM glucose measures 1.5 h after exercise. Circulating plasma catecholamines are reported to increase during high-intensity exercise which leads to a 7–8-fold increase in hepatic glucose production compared to moderate intensity exercise (Marliss and Vranic, 2002). During recovery, catecholamine concentrations rapidly decrease, removing the catecholamine inhibition of glucose-stimulated insulin secretion (Marliss and Vranic, 2002). This leads to elevated plasma glucose and insulin levels after high-intensity exercise compared to moderate intensity exercise. This stress hormonal response may explain the comparatively higher glucose levels immediately after LV-HIIE, and higher post-exercise insulin levels throughout the recovery period, compared to CMIE. It is important to note that this elevated glucose response with LV-HIIE was transient and did not negatively impact the 24-h improvements in glycemic control post-exercise.

Limitations and Strengths

A strength of this study was the recruitment of inactive and overweight and obese men and women including a subgroup of women with PCOS. Although, sex differences in glycemic control (Gillen et al., 2014) and postprandial oxidative stress have been reported (Bloomer and Lee, 2013), sample size and participant characteristics were well matched between LV-HIIE and CMIE exercise groups, and all females were tested in the early follicular phase of the menstrual cycle. A small sample size prevented subgroup analysis to explore whether particular groups (men, women, and/or women with PCOS) had different glycemic

control responses to the exercise sessions. Despite this limitation, this study provides preliminary data to suggest that overweight and obese men, women, and women with PCOS, may be able to improve 24-h glycemic control with a single session of CMIE or LV-HIIE. Future research is warranted to investigate glycemic responses in these subgroups. Plasma TBARS (malondialdehyde) and hydrogen peroxide have previously been used to reflect systemic postprandial oxidative stress (Bloomer et al., 2010; Bloomer and Lee, 2013; Canale et al., 2014). Nevertheless, future research would benefit from employing additional measures of oxidative stress in plasma such as the oxidized/reduced glutathione ratio or the direct measurement of ROS through spin trapping and electron spin resonance spectroscopy. Another potential study limitation is that the participants were not blinded to real-time CGM readings, which when prompted are displayed on an LCD screen. If possible, future studies should adopt a CGM system such as the iPro®2 Professional CGM (Medtronic MiniMed, Northridge, CA) which allows blinding of participants to real-time CGM readings. A potential limitation of the study is the natural variability of outcome measures between the two exercise groups which is inherent to a parallel design study. Despite appropriate matching of participant characteristics and the inclusion of the rest-control day for exercise day comparisons, future research would benefit from adopting a cross-over design study to confirm these findings. In summary, further cross-over randomized control trials are warranted to explore the acute and chronic impacts of LV-HIIE in larger groups of people in specific clinical populations, and using more diverse exercise protocols including that of work-matched CMIE.

CONCLUSIONS

A single session of CMIE, but not LV-HIIE, attenuated postprandial plasma hydrogen peroxide and glycemia when performed 1 h after breakfast consumption. Yet, over the 24-h post-exercise period LV-HIIE elicited similar improvements in glycemic control to CMIE. Given its time-efficient nature, LV-HIIE may be an effective exercise mode to incorporate into exercise programs for the improvement of 24-h glycemic control in inactive, overweight and obese adults. Furthermore, measuring glycemic control immediately after exercise may not accurately reflect functional improvements over the 24-h post-exercise recovery period, especially with respect to high-intensity exercise.

AUTHOR CONTRIBUTIONS

LP, CS, LB, IL, KH, AM, and NS contributed to the study design and acquirement of ethical approval. LP, CS, LB, and NS contributed to data collection. LP analyzed the data and drafted the initial manuscript. The remaining authors critically revised the manuscript. All authors approved the final version of the manuscript. NS, LP are guarantors of the manuscript and take full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

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The following research study and subsequent publication was not part of the PhD. However, data interpretation, manuscript drafting, and subsequent publication was conducted during the PhD candidature. This publication was not a requirement for a previous degree (i.e. Honours or Masters) and due to the relevance to the current thesis has been included as an appendix for the reader's perusal.

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Influence of exercise intensity on systemic oxidative stress and antioxidant capacity

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Summary

The aim of the current study was to examine the influence of exercise intensity on systemic oxidative stress (OS) and endogenous antioxidant capacity. Non-smoking, sedentary healthy adult males (n = 14) participated in two exercise sessions using an electronically braked cycle ergometer. The first session consisted of a graded exercise test to determine maximal power output and oxygen consumption (VO_{2max}). One week later, participants undertook 5-min cycling bouts at 40%, 55%, 70%, 85% and 100% of VO_{2max}, with passive 12-min rest between stages. Measures of systemic OS reactive oxygen metabolites (dROM), biological antioxidant potential (BAP), heart rate (HR), VO_2, blood lactate and rating of perceived exertion were assessed at rest and immediately following each exercise stage. Significant ($P<0{\cdot}05$) differences between exercise bouts were examined via repeated measures ANOVA and post hoc pairwise comparisons with Bonferroni correction. Increasing exercise intensity significantly augmented HR ($P<0{\cdot}001$), VO_2 ($P<0{\cdot}001$), blood lactate ($P<0{\cdot}001$) and perceived exertion ($P<0{\cdot}001$) with no significant effect on dROM levels compared with resting values. In contrast, increasing exercise intensity resulted in significantly ($P<0{\cdot}01$) greater BAP at 70% (2427 ± 106), 85% (2625 ± 121) and 100% (2651 ± 92) of VO_{2max} compared with resting levels (2105 ± 57 μmol Fe^{2+}/L). The current results indicate that brief, moderate-to-high-intensity exercise significantly elevates endogenous antioxidant defences, possibly to counteract increased levels of exercise-induced reactive oxygen species. Regular moderate-to-high-intensity exercise may protect against chronic OS associated diseases via activation, and subsequent upregulation of the endogenous antioxidant defence system.

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Introduction

Throughout everyday life, the body constantly undergoes redox reactions that involve reactive oxygen species (ROS) and antioxidants (Halliwell, 1991). ROS can react with and remove electrons from normal structural and functioning proteins causing cellular damage (Halliwell, 1991). In contrast, reducing compounds such as antioxidants have an affinity towards donating their electrons to other cells enabling them to neutralize/scavenge ROS or inhibit its production (Halliwell, 1991).

While modest increases in ROS production are regulated by the body's endogenous antioxidant defence system, which include both enzymatic and non-enzymatic sources, certain ROS promoting stimuli such as exercise, diet, age and disease may prove overpowering and lead to a redox balance shift in favour of oxidative stress (OS) (Valko *et al.*, 2007). OS has been associated with both beneficial and harmful effects on health, and as such recent classifications have been redefined to identify OS as 'a redox imbalance resulting in increased levels of ROS production and/or oxidant biomarkers' irrespective of the repercussions (Nikolaidis *et al.*, 2012). OS has been implicated to play a primary and/or secondary role with over 100 acute and chronic human diseases such as diabetes, hypertension, pre-eclampsia, atherosclerosis, acute renal failure and Alzheimer and Parkinson diseases (Valko *et al.*, 2007). Although excess ROS results in the oxidation of proteins, lipids and DNA, recent studies indicate ROS to be a beneficial and necessary requirement for optimal physiological functioning (Radak *et al.*, 2013). For example, ROS act as signalling molecules for mitochondrial biogenesis, endogenous antioxidant upregulation, insulin signalling, angiogenesis,

neurogenesis, epigenetics and various immune and inflammatory recovery processes (Radak *et al.*, 2013). The diametric effects of ROS/OS are suggested to be influenced by the 'biological context' in which they take place. As such the biological outcomes are largely dependent upon not only the degree of ROS exposure, but also the exposure time, the type of ROS, the spatial distribution of ROS, the organs and organelles involved, and whether endogenous antioxidant defences are overwhelmed (Bashan *et al.*, 2009). For example, ROS are involved in both the physiological and pathological functioning of insulin signalling and insulin resistance, with the 'biological context' arbitrating the beneficial or harmful biological outcome (Bashan *et al.*, 2009).

Over the past 30 years, numerous studies have demonstrated that acute aerobic and anaerobic exercise, both maximal and submaximal, can elicit OS through excess exercise-induced ROS production (Fisher-Wellman & Bloomer, 2009). Exercise-induced ROS are transient and have been reported to instigate many redox-specific health adaptations (Radak *et al.*, 2013). In contrast, lifestyle factors such as excess energy intake and sedentary behaviour have been reported to induce chronic levels of ROS and result in pathological responses such as insulin resistance (Fisher-Wellman & Neufer, 2012) and other chronic lifestyle diseases (Valko *et al.*, 2007), many of which may be alleviated by regular exercise-induced redox adaptations (Radak *et al.*, 2013). While exercise-induced oxidative stress (EIOS) has received significant attention (Fisher-Wellman & Bloomer, 2009), the relationship between exercise subcomponents, in particular that of exercise intensity and the resultant production of ROS remains unclear. High-intensity exercise has been shown to promote similar, and in some cases, greater metabolic health benefits than that of moderate-intensity exercise (Gibala *et al.*, 2012), potentially mediated through exercise-induced ROS (Kang *et al.*, 2009); however, research investigating exercise intensity and OS is limited. With the current understanding of exercise and its potential to improve general health and chronic disease through beneficial redox alterations, the impact of exercise intensity on OS, ROS and antioxidant capacity remains pivotal in advancing exercise prescription for enhanced health.

To date, the majority of studies examining OS have focused on single intensity protocols, a wide range of exercise modes, durations and OS/antioxidant biomarkers (Fisher-Wellman & Bloomer, 2009). Relatively few studies have manipulated exercise intensity while keeping the mode of exercise and duration constant, resulting in a lack of definitive conclusion regarding the role of exercise intensity and its inducement of OS and antioxidant status (Fisher-Wellman & Bloomer, 2009). Current findings suggest that high-intensity exercise (>75% maximal aerobic capacity, VO_{2max}) promotes greater OS in humans compared with low-moderate-intensity exercise (25–70% VO_{2max}) (Lovlin *et al.*, 1987; Leaf *et al.*, 1997; Goto *et al.*, 2003, 2007). Unfortunately, a large number of these studies have not examined total antioxidant defence capacity (TAC) and thus limits the scope of their findings (Lovlin *et al.*, 1987; Leaf *et al.*, 1997; Goto *et al.*, 2003, 2007; Hoffman *et al.*, 2007). As EIOS can upregulate endogenous antioxidant enzymes (Ristow *et al.*, 2009), which may protect against chronic OS-related diseases (Valko *et al.*, 2007; Fisher-Wellman & Neufer, 2012; Radak *et al.*, 2013), a greater examination of both OS and antioxidant status is required to clarify the role of EIOS in health and disease. Preliminary research has reported higher-intensity exercise to elicit greater TAC responses (Schneider *et al.*, 2005; Daud *et al.*, 2006; Tyldum *et al.*, 2009); however, only a few studies exist and a limited number of exercise intensities have been investigated. As a result, optimal EIOS and TAC exercise intensity thresholds have not been adequately investigated. Subsequently, the aim of the current study was to examine the relationship between exercise intensity and its inducement of OS and TAC. It was hypothesized that increasing exercise intensity would result in greater OS and TAC with the possibility of the existence of a threshold for significant increases in OS and TAC, similar to that observed for other metabolic products such as lactic acid [i.e. lactate threshold (Acevedo & Goldfarb, 1989)].

Methods

Participants

Non-smoking, healthy adult males ($n = 14$; mean ± SEM age, height, mass, BMI and body fat percentage of 22·0 ± 1·0 years, 179·4 ⊥ 2·0 cm, 81·4 ⊥ 2·0 kg, 25·4 ⊥ 0·7 kg m^{-2} and 17·9 ± 1·0%, respectively) volunteered for this study. A priori power analyses (power 90%, α at 0·05) using previously published data (Martarelli *et al.*, 2011) indicated that sample sizes of $n = 10$ and $n = 8$ were required to identify substantial practical changes in OS (via reactive oxygen metabolite assay) and TAC [via BAP assay], respectively. All participants were sedentary and had not participated in regular, moderate-to-high levels of physical activity within the past 3 months. Prior to participation, all participants completed a prescreening medical health history questionnaire and provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki for experiments involving humans and ethical approval obtained from the James Cook University Human Ethics Subcommittee (approval H3257). Participants abstained from food (6 h) and physical activity, alcohol and caffeine consumption (24 h) prior to testing and were not currently taking any medications or supplements. Testing consisted of an incremental cycle VO_{2max} determination test followed by an intermittent cycle protocol approximately 1 week later.

Incremental cycle VO_{2max} determination

Participants completed an incremental cycle protocol to determine VO_{2max} and maximum work load (W_{max}) using an electromagnetically braked cycle ergometer (Lode Excalibur; Lode, Groningen, the Netherlands). The incremental protocol

consisted of a 5-min warm-up at 70 W (80 revolutions per minute, rpm), 2 min of passive rest, and an initial workload of 75 W increasing 25 W every 3 min until participants were unable to maintain the required cadence (within 5 rpm). HR was measured using a telemetric HR monitor (RS800; POLAR, Kupio, Finland) every minute along with rating of perceived exertion (RPE). Ventilatory expired gas (10 s averages) was collected from each participant and analysed using a metabolic cart (VIASYS Healthcare, Inc., SensorMedics, Yorba Linda, CA, USA) that was calibrated in accordance with the manufacturers guidelines. Determination of VO_{2max} was based on achievement of at least three of the following criteria: VO_{2max} plateau (successive VO_2 values <2 ml kg^{-1} min^{-1}); attainment of a HR within ten beats of age-predicted maximum HR; respiratory exchange ratio >1·10, RPE > 18, blood lactate >8 mmol l^{-1}; and volitional exhaustion (Midgley *et al.*, 2007). Maximum workload (W_{max}) was determined as the workload eliciting VO_{2max}.

Intermittent cycle protocol

Approximately 1 week after the completion of the VO_{2max} determination, participants completed an intermittent cycle protocol with the same electronically braked cycle ergometer and metabolic cart used during the VO_{2max} determination. The intermittent protocol involved 5 min of steady state cycling (80 rpm) at 40%, 55%, 70%, 85% and 100% of VO_{2max} separated by 12 min of passive seated rest to allow for the immediate analysis of blood samples. Exercise intensities were calculated based on the VO_{2max} determination and confirmed via respiratory gas analysis. HR and RPE were recorded every minute of the protocol with finger-tip capillary blood samples (approximately 100 µl) collected at rest and the end of each bout for the analysis of lactate, OS and TAC.

Assessment of blood samples

Blood lactate was measured via capillary blood samples using a hand-portable lactate analyser (Accutrend; Roche Diagnostics, Mannheim, Germany). Capillary blood was collected in lithium heparin tubes, centrifuged at 1800 *g* for 10 min and aliquots of plasma collected for immediate analysis of OS and TAC using the Free Radical Analytical System (FRAS 4; H&D srl, Parma, Italy). The FRAS4 system has been reported to produce a simple, accurate and reproducible assessment of systemic OS and TAC (Iamele *et al.*, 2002; Trotti *et al.*, 2002; Cornelli *et al.*, 2011).

Determination of total antioxidant capacity

Total antioxidant capacity was measured using the BAP assay, which measures the plasma's ability to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions. Using a special chromogenic substrate that changed colour intensity during an oxidation–reduction reaction, plasma antioxidant potential was photometrically measured (absorption at 505-nm wavelength) using the FRAS4 system with values expressed in µmol Fe^{2+}/L. Values of ≥2200 µmol Fe^{2+}/L reflect normal TAC and antioxidant defence (Trotti *et al.*, 2002).

Determination of oxidative stress

Systemic OS was measured using the reactive oxygen metabolites (dROM) assay, which quantified total plasma hydroperoxide, oxidative derivatives of phospholipids (and lipids), proteins and DNA. In the presence of iron (reagent provided with assay kit), hydroperoxides reacted to generate alkoxyl and peroxyl radicals that oxidized N,N-diethyl-p-phenylenediamine (reagent provided with assay kit) to produce a pink-coloured derivative that was photometrically quantified (absorption at 505-nm wavelength) by the FRAS4 system. Values were expressed in U CARR (Carratelli units) with 1 U CARR equating to 0·08 mg dl^{-1} of hydrogen peroxide. dROM values >300 U CARR indicate OS with higher values indicating higher levels of OS (Trotti *et al.*, 2002). The dROM assay has been validated with electron spin resonance spectroscopy (Alberti *et al.*, 2000), the current gold standard for OS measurement with dROM and BAP analyses used extensively for the measurement of EIOS (Andersson *et al.*, 2010; Martarelli *et al.*, 2011; Taito *et al.*, 2013). Previously, the dROM coefficient of variation (CV) was reported to be much smaller (9%) than other OS markers such as isoprostanes, oxidized DNA and carbonylated proteins (61%, 57% and 65%, respectively) (Cornelli *et al.*, 2011) with the CV for BAP and dROM being <4% and <6%, respectively, in our laboratory.

Statistical analysis

Statistical analyses were conducted using Predictive Analytics Software (PASW v18; SPSS Inc., Chicago, WI, USA). Data were confirmed via Shapiro–Wilk tests as normally distributed and presented as mean ± SEM. Comparisons between rest and exercise intensities were examined using repeated measures ANOVA and post hoc pairwise comparisons with a Bonferroni correction. A P-value of <0·05 was deemed statistically significant.

Results

Participants completed the incremental cycle protocol with VO_{2max} confirmed in accordance with the defined criteria. Physiological and perceptual responses after the final stage of the incremental cycle protocol are presented in Table 1.

All participants completed the intermittent exercise intensity protocol. Each bout was confirmed within 4% of the estimated exercise intensity with all bouts significantly different to each other in terms of VO_{2max} (Table 2). Increasing exercise intensity had no significant effect (P>0·05) on dROM levels compared with resting values while increasing exercise intensity

Table 1 Maximal physiological and perceptual responses at the end of the incremental cycle VO_{2max} determination.

	Mean ± SEM
Lactate (mmol l^{-1})	10·4 ± 0·7
Heart rate (bpm)	185 ± 3
Rating of perceived exertion	19·8 ± 0·3
W_{max} (W)	265 ± 11
VO_{2max} (ml kg^{-1} min^{-1})	42·6 ± 2·1

resulted in a significantly greater BAP at 70% ($P = 0·009$), 85% ($P = 0·005$) and 100% ($P<0·001$) of VO_{2max} compared with resting levels (Fig. 1). HR, RPE, blood lactate and VO_2 were significantly increased with increasing exercise intensity (Table 2).

Discussion

The findings of the present study indicated that EIOS, as reflected by dROM, was unaltered with acute bouts of increasing exercise intensity and no existence of an EIOS threshold. In contrast, acute high-intensity exercise (≥70% VO_{2max}) significantly increased plasma TAC compared to that of lower-intensity exercise. These findings provide novel support for the use of high-intensity exercise in prescribing exercise for the improvement and/or maintenance in health, particularly in diseased populations whom are susceptible to chronic OS.

The current results support the findings of Schneider *et al.* (2005) who reported TAC to significantly increase with high-intensity treadmill exercise compared with low-moderate-intensity exercise with no changes occurring in lipid peroxidation. In contrast, several studies have reported exercise intensity, predominantly that of high intensity, to induce greater levels of OS compared with lower intensities (Lovlin *et al.*, 1987; Leaf *et al.*, 1997; Goto *et al.*, 2003, 2007). It was previously suggested that exercise must be exhaustive to overwhelm endogenous antioxidant defences and promote a state of OS (Vina *et al.*, 2000); however, this seems unnecessary as a recent review has identified numerous studies showing significant increases in OS biomarkers with non-exhaustive exercise (Fisher-Wellman & Bloomer, 2009). More recently, it has been suggested that exercise volume (duration x intensity) and whether endogenous antioxidant defences are overwhelmed, were the primary mediators of EIOS (Fisher-Wellman & Bloomer, 2009). The significant increases in TAC and lack of concurrent changes in OS levels in the present study suggest that exercise volume may not have been great enough to exhaust endogenous antioxidant reserves and thus redox homeostasis was maintained, a finding also reported by others (Schneider *et al.*, 2005; Andersson *et al.*, 2010). In addition, the disparity between the current and prior

Table 2 Physiological and perceptual responses at rest and at the completion of each exercise intensity bout.

	Rest	40% VO_{2max}	55% VO_{2max}	70% VO_{2max}	85% VO_{2max}	100% VO_{2max}
Heart rate (bpm)	66 ± 3	113 ± 4[a]	132 ± 6[a]	153 ± 6[a,b]	168 ± 4[a,b,d]	182 ± 6[a,b,c,d]
Rating of perceived exertion		8·4 ± 0·5	10·8 ± 0·6	13·1 ± 0·6[b]	16·4 ± 0·6[b,c,d]	19·5 ± 0·3[b,c,d]
Lactate (mmol l^{-1})	2·2 ± 0·2	3·2 ± 0·4	3·6 ± 0·4	5·4 ± 0·5[a]	8·3 ± 0·6[a,b,c,d]	10·8 ± 0·7[a,b,c,d,e]
VO_2 (ml kg^{-1} min^{-1})		18·6 ± 0·7	23·4 ± 0·9[b]	29·3 ± 1·2[b,c]	35·2 ± 1·5[b,c,d]	40·9 ± 1·3[b,c,d,e]
Relative workload (%VO_{2max})		43·8 ± 1·4	54·9 ± 1·3[b]	68·9 ± 1·9[b,c]	83·0 ± 2·8[b,c,d]	96·6 ± 2·3[b,c,d,e]

Values are mean ± SEM; [a]$P<0·05$ versus rest, [b]$P<0·05$ versus 40%, [c]$P<0·05$ versus 55%, [d]$P<0·05$ versus 70%, [e]$P<0·05$ versus 85%.

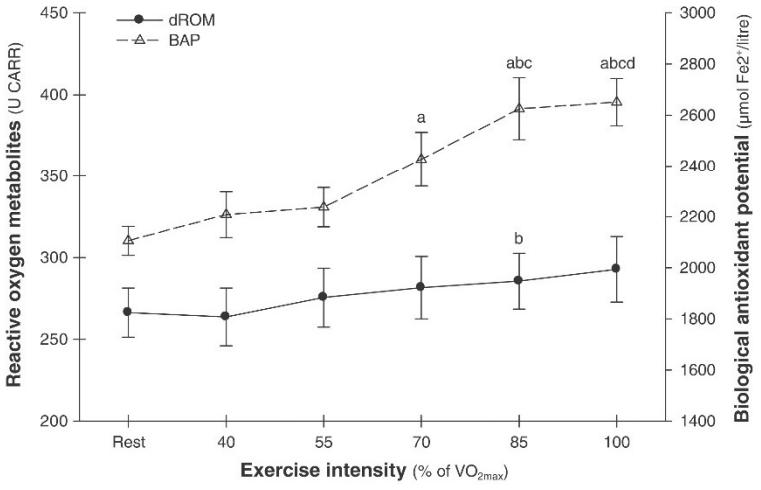

Figure 1 Reactive oxygen metabolite (dROM) and biological antioxidant potential (BAP) responses at the completion of each exercise intensity bout. Values are mean ± SEM. [a]$P<0·05$ versus rest, [b]$P<0·05$ versus 40%, [c]$P<0·05$ versus 55% and [d]$P<0·05$ versus 70%.

results may be due to differences in exercise protocol duration [5 min versus 30 min of cycling (Goto *et al.*, 2003, 2007)], exercise mode [cycling versus treadmill (Leaf *et al.*, 1997)], and the biomarkers analysed [dROMS versus malondialdehyde-modified low density lipoprotein (Goto *et al.*, 2007), 8-isoprostane (Goto *et al.*, 2003, 2007) and exhaled ethane and pentane (Leaf *et al.*, 1997)], factors previously identified as contributors to interstudy differences (Fisher-Wellman & Bloomer, 2009). As previously discussed, EIOS is likely a product of exercise volume, and considering that exercise volume in the current study was considerably lower than previous studies it is not surprising that dROM levels remained relatively unchanged. In support, Andersson *et al.* (2010) reported no changes in dROM during a 90-min soccer game (average intensity 82 ± 3% peak HR) in elite female soccer players while acute antioxidant status (indicated by uric acid, total glutathione, α-tocopherol and ascorbic acid levels) significantly increased. The authors concluded that endogenous antioxidant defences were adequate in maintaining redox homeostasis during short bouts of intermittent exercise, a finding in agreement with the current study and others who have indicated healthy individuals to possess adequate antioxidant defences in maintaining redox homeostasis during exercise (Peternelj & Coombes, 2011). The current findings contribute to the growing trepidation of unnecessary antioxidant supplementation in healthy individuals whom already possess adequate endogenous antioxidant defences (Peternelj & Coombes, 2011). Indeed, accumulating evidence suggests supplementation may potentially blunt certain beneficial exercise-induced adaptations including antioxidant defence upregulation (Gomez-Cabrera *et al.*, 2008; Ristow *et al.*, 2009).

In the current study, dROM were not significantly altered with an increase in TAC occurring at higher intensities during intermittent exercise. These results may reflect an increase in ROS production, stimulating the release of plasma antioxidants and subsequent ROS scavenging with higher-intensity exercise. Subsequently, high-intensity exercise may induce greater redox-associated health adaptations via upregulation of endogenous antioxidant defences (Radak *et al.*, 2013). Further research will clarify the role of high-intensity exercise in the upregulation of antioxidant defence and other redox-mediated health adaptations.

While ROS mediate many beneficial health adaptations, excessive ROS exposure can overwhelm endogenous antioxidant defences and result in chronic OS, a state associated with over 100 chronic diseases including the functional decline observed with ageing (Valko *et al.*, 2007). Considerable research has shown chronically increased dROM levels and lowered BAP levels in diseased populations (Markoulis *et al.*, 2006; Kotani *et al.*, 2008; Ishii *et al.*, 2011; Naruse *et al.*, 2013) and those with known risk factors (Atabek *et al.*, 2004; Kamezaki *et al.*, 2008; Kotani *et al.*, 2008; Fukui *et al.*, 2011; Naruse *et al.*, 2013). It is therefore not surprising that sedentary, overweight and diseased populations exhibit compromised antioxidant defences and subsequent higher chronic OS levels then their healthier counterparts (Fisher-Wellman *et al.*, 2009). Our results indicate an important influencing role of exercise intensity on global OS and endogenous antioxidant levels, which may have long-term health benefits for sedentary and other populations susceptible to excess OS. Indeed chronic exercise has been reported to upregulate many endogenous antioxidant enzymes such as superoxide dismutase 1 and 2, glutathione peroxidase and catalase (Gomez-Cabrera *et al.*, 2008; Ristow *et al.*, 2009). Furthermore, older adults who underwent twelve weeks of low-level physical activity (100 min per week) significantly enhanced TAC (indicated by BAP, thioredoxin and glutathione peroxidase levels) while attenuating dROM levels ($P = 0{\cdot}05$) compared with non-exercising controls (Takahashi *et al.*, 2013). Likewise, Kurban *et al.* (2011) reported 12 weeks of moderate-intensity power walking to significantly enhance TAC in type 2 diabetics compared with non-exercising controls. While low-moderate-intensity exercise has been reported to promote beneficial redox adaptations in healthy and unhealthy populations, the impact of high-intensity exercise on redox-mediated adaptations remains more elusive.

High-intensity exercise programmes have been gaining popularity (Gibala *et al.*, 2012); however, very little is currently known about the potential of these programmes to induce changes in redox homeostasis. The results of the current study support the limited but growing notion that high-intensity exercise may induce beneficial redox homeostasis alterations and greater health benefits than that of low-moderate-intensity exercise (Tyldum *et al.*, 2009; Fisher *et al.*, 2011; Gabriel *et al.*, 2012; Gibala *et al.*, 2012; Bogdanis *et al.*, 2013). Previously, high-intensity exercise training, particularly interval training, has been reported to elicit comparable and/or greater metabolic and cardiovascular improvements in glycaemic control, exercise performance, VO_{2max}, resting glycogen content, lipid oxidation, vascular endothelial function and increased mitochondrial biogenesis (Gibala *et al.*, 2012) compared with lower exercise intensities, potentially through various redox sensitive pathways (Kang *et al.*, 2009; Radak *et al.*, 2013). Thus, high-intensity exercise may play a crucial mediating role in promoting health benefits through redox alterations. Several studies have reported acute single and multiple high-intensity exercise bouts to enhance TAC, endothelial function, and attenuate postprandial OS, sometimes to a greater extent than moderate continuous exercise or non-exercising controls (Tyldum *et al.*, 2009; Gabriel *et al.*, 2012). Furthermore, short duration high-intensity interval training has also been shown to significantly enhance endogenous antioxidant defence and reduce subsequent EIOS (Fisher *et al.*, 2011; Bogdanis *et al.*, 2013). These studies indicate the potential for high-intensity exercise to promote greater redox-related health benefits compared with lower-intensity exercise. Our results support and expand upon these findings by identifying that 5 min cycling bouts at 70% VO_{2max} and above were adequate exercise stimuli to significantly increase circulating plasma antioxidants with the greatest increase in TAC occurring at ≥85% VO_{2max}. Conversely, short cycling bouts at 40% and

50% VO_{2max} showed no changes in TAC and thus may not be suitable exercise stimuli for promoting the upregulation of TAC. Furthermore, previous research has primarily investigated physically active or trained individuals (Tyldum *et al.*, 2009; Fisher *et al.*, 2011; Gabriel *et al.*, 2012; Bogdanis *et al.*, 2013) with the current results among the first to indicate that healthy but inactive populations may also receive similar beneficial redox alterations from high-intensity exercise. Current and previous findings (Tyldum *et al.*, 2009; Gabriel *et al.*, 2012) indicate that exercise prescription aimed to improve endogenous antioxidant capacity and protect against chronic OS and associated diseases may benefit from 5-min cycling intervals at moderate-high-intensity exercise (≥70% VO_{2max}). Whether interval repetition and/or recovery period length influences TAC is yet to be determined.

It should be noted that the current study was limited to an examination of apparently healthy adult males and included a global measure of OS (i.e. dROM). Males were specifically examined to reduce any potential effect of hormonal influences on OS (Bloomer & Fisher-Wellman, 2008) with further studies needed to confirm the current results for females. OS measures were limited to a single, valid and reliable biomarker (dROM). Thus, it is possible that oxidative damage may have occurred but was not detected by the assay. This is unlikely however as the dROM assay measures combined oxidative damage to proteins, lipids and DNA and thus provides a dependable measurement of the global OS condition (Trotti *et al.*, 2002). On the other hand, many common OS biomarkers assess only their specific derivative of oxidative damage (protein, lipids or DNA) and exhibit higher coefficients of variation with OS levels often over/underestimated (Cornelli *et al.*, 2011). Lastly, the exercise intensities undertaken were conducted within the same session with a brief (12-min) rest between bouts that may have not been sufficient to return OS and TAC levels to baseline. Subsequently, the increased TAC may have reflected an accumulation effect of the bouts. However, increases in TAC without changes in OS have been reported following a single bout of high-intensity (>77% VO_{2max}) exercise (Schneider *et al.*, 2005). Future studies may extend the current results by examining exercise bouts on separate days to clarify further the impact of exercise intensity on redox balance.

Conclusion

Our results indicate that high-intensity exercise of short duration significantly increases endogenous antioxidant levels compared with rest and lower-intensity exercise while not incurring significant OS. Increased TAC, possibly indicative of a high transient increase in ROS, suggests that higher-intensity exercise may promote greater redox health benefits and may help protect against chronic OS-related diseases. Considering that 'lack of time' is one of the most commonly cited barriers to achieving recommend physical activity guidelines (Sequeira *et al.*, 2011), high-intensity exercise such as interval training may be a viable model to improve and/or maintain endogenous antioxidant defences and general well-being, particularly in sedentary individuals that are susceptible to chronic levels of OS. Further research will elucidate the long-term effects and potential mechanisms of high-intensity exercise and redox-mediated health adaptations.

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Conflict of interest

The authors declare that they have no conflict of interest for this study. Anthony Leicht is a Scientific Board member of the International Co-operation Oxidative Balance, which is sponsored by H&D srl (manufacturer of the FRAS 4 system).

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Appendix C: Western blot methods.

Muscle samples were obtained from the vastus lateralis under local anaesthesia (Xylocaine 1%) utilizing a Bergström needle with suction (113). Western blot procedures were conducted in accordance with recommendations outlined by Murphy and Lamb (296). Phosphorylation and abundance of specific proteins in whole muscle lysate were determined with all constituents present (i.e. no centrifugation). Centrifugation of lysate, which typically removes the nuclei, mitochondria, and cell debris, was avoided due to the previously reported loss of total cellular protein that can occur (296, 297). Furthermore, the western blot method adopted used SuperSignal West Femto (Pierce, Rockford, IL) ultra-sensitive enhanced chemiluminescent to allow for lower total protein content loading and better linearity and sample resolution (292, 296).

Thirty cryosections of skeletal muscle (20 µm) were cut (Cryostat HM550, Thermo Scientific, Australia) at -20°C and added to Eppendorf tubes containing 300 µl of homogenising buffer (0.125M TRIS-HCL [pH 6.8], 4% SDS, 10% Glycerol, 10mM EGTA, 0.1M DTT, and with 0.1% v/v protease and phosphatase inhibitor cocktail [#P8340 and #P5726, Sigma Aldrich]). Samples were rotated for 1 hour at room temperature, vortexed and frozen at -80°C. Samples were then thawed on ice and total protein content of muscle lysate was determined using the commercially available Red 660 Protein Assay kit with SDS neutralizer as per the manufacturer's instructions (Red 660, G-Biosciences, St. Louis, MO, USA). Eight µg of protein for each sample was prepared in 3 µl of Bromophenol blue (1%), heated for 5 minutes at 95°C, and loaded into Criterion™ TGX™ Pre-Cast Midi Gels (Biorad).

Proteins were than separated by electrophoresis (Criterion™ Vertical Electrophoresis Cell, Biorad) at 200 volts for 45 minutes with electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). The separated proteins were then transferred to a pre-assembled PVDF membrane (Trans-Blot Turbo PVDF Transfer Pack, Bio-Rad, Richmond, CA) with a Trans-Blot Turbo Transfer System (Bio-Rad) using the pre-set setting for mixed molecular weight protein. Membranes were blocked with Tris-Buffered Saline-Tween (TBST: 0.1 M Tris Base, 1.5 M NaCl, 0.1% Tween-20) and 5% skim milk

for 1 hour and then washed (3 x 5 minutes) with TBST. Membranes were incubated at 4°C overnight in 10 ml of relevant primary antibody solution with gentle shaking. After incubation membranes were washed with TBST (3 x 5 minutes), incubated for 1 hour at room temperature with appropriate dilutions of horseradish peroxidase conjugated secondary antibody in 5% skim milk and TBST. Membranes were then re-washed with TBST (3 x 5 minutes) and incubated in SuperSignal West Femto Maximum Sensitivity (Pierce, Rockford, IL) substrate for 5 minutes prior to imaging with a ChemiDoc™ MP System (Bio-Rad). Densitometry values were measured using Image Lab software (Bio-Rad) with background correction (rolling disc method) and exported to excel. After imaging, membranes were washed briefly in TBST, stained with 0.1% Brilliant Blue R-350 (Sigma Aldrich, St. Louis, MO) in 1:1 methanol/distilled water (dH_2O) solution for 3 min, de-stained in 1:5:4 ethanol/acetic acid/dH_2O solution for 40 seconds, and rinsed with dH_2O and air-dried for ~1 hour prior to imaging in the ChemiDoc™ MP System. All densitometry values are expressed relative to an internal standard (pooled lysate sample) and normalized to the total protein content of each lane obtained from the modified Coomassie staining protocol. Where appropriate, phosphorylated proteins are expressed relative to specific total protein content. Arbitrary densitometry values are expressed as a relative fold change to baseline/control trial values.

Appendix D: Plasma analysis.

Venous blood was collected in collection tubes containing ethylenediaminetetraacetic acid (EDTA), separated into plasma by centrifugation (10 min at 3500 rpm, 4°C), then aliquoted and stored at -80°C until analysed. All assay buffers, reagents and appropriate standards were prepared as per the manufacturer's instructions using the provided contents in the respective commercial assay kits. All plasma samples were thawed on ice and vortexed appropriately prior to analysis. The intra-assay coefficient of variation (CV) for each study was determined by averaging the CV of each duplicate for all samples analysed (e.g. all time-points for all participants). The inter-assay CV for each study was determined by averaging the CV for each assay standard between each 96 well plate (3 plates for Chapter 3, 2 Plates for Chapter 4, and 5 plates for Chapter 5.)

Plasma catalase activity (Cayman Chemical).

After the samples were thawed, 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of formaldehyde standards, samples and/or diluted positive control (bovine liver catalase) were added to 96 well plates in duplicate. The reaction was initiated by adding 20 µl of diluted Hydrogen Peroxide as quickly as possible to all the wells and the precise time of addition was noted. The plate was covered with a plate cover and incubated on an orbital shaker for 20 minutes at room temperature. 30 µl of diluted Potassium Hydroxide was then added to each well to terminate the reaction followed by the addition of 30 µl of Catalase Purpald to each well. The cover plate was then covered and incubated for 10 minutes at room temperature on an orbital shaker. 10 µl of Catalase Potassium Periodate was then added to each well and the plate covered and incubated for five minutes at room temperature on an orbital shaker. The absorbance was then read at 540 nm using a spectrophotometer (xMark microplate spectrophotometer, Bio-Rad Laboratories, Mississauga, ON, Canada). Raw data was exported to Microsoft Excel. The background absorbance value (sample buffer only) was subtracted from all sample, standard and positive control values. Catalase activity for each sample was determined using the equation derived from the standard curve as per the manufacturer's instructions. One unit of catalase activity is defined as the amount of enzyme required to cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.

Plasma thiobarbituric acid reactive substances (Cayman Chemical).

After thawing, 100 µl of sample or standard, and 100 µl of TCA Assay Reagent (10%) were added to a 1.5ml Eppendorf tube and lightly vortexed to mix. 800 µl of the Colour Reagent was then added to each tube and vortexed. to each vial and vortex. Samples when than capped, and placed in a custom-made foam holder and boiled vigorously for one hour in a water bath. After one hour of boiling, the tubes were immediately removed and placed in an ice bath for 10 minutes to stop the reaction. After 10 minutes in the ice bath, the tubes were centrifuged for 10 minutes at 1,600 x g at 4°C. 200 µl of each sample and standard were carefully removed from the tubes and transferred to a clear 96 well microplate in duplicate. The absorbance was measured at 540 nm and the

raw data exported to Microsoft Excel. The background absorbance (Milli-Q water) was subtracted from all samples and standards. Malondialdehyde concentration of each sample was determined using the equation derived from the standard curve as per the manufacturer's instructions. Plasma TBARS are expressed as nmol/ml of Malondialdehyde equivalents.

Plasma hydrogen peroxide (Amplex UltraRed assay; Molecular Probes).

After thawing, 50 µl of each sample and standards were transferred to a clear 96 well microplate in duplicate. 50 µl of the Amplex® Red reagent/HRP working solution was added to each well and incubated at room temperature away from light for 30 minutes. Absorbance at 560 nm was immediately measured and the raw values exported to Microsoft Excel. Background absorbance (no-hydrogen peroxide control) was subtracted from all samples and standards. Plasma hydrogen peroxide concentration of each sample was determined using the equation derived from the standard curve as per the manufacturer's instructions. Plasma hydrogen peroxide concentration is expressed as nmol/ml.

Plasma superoxide dismutase activity (Cayman Chemical).

After thawing, 200 µl of the diluted Radical Detector and 10 µl of each sample and standard were added to a clear 96 well microplate in duplicate. The reaction was initiated by adding 20 µl of diluted Xanthine Oxidase as quickly as possible to all the wells and the precise time was noted. The 96-well plate was then carefully shaken for a few seconds to mix, and then covered and placed on an orbital shaker for 20 minutes at room temperature. The absorbance was then read at 460 nm using a plate reader and raw values exported to Microsoft Excel. Background absorbance (sample buffer) was subtracted from all samples and standards and the linearized rate of the superoxide dismutase standards and samples were calculated as per the manufacturer's instructions. Plasma superoxide dismutase activity for each sample was than determined by substituting the linearized rate using the equation derived from the standard curve. One unit of SOD activity is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Plasma insulin (HI-14K, Millipore).

After thawing of samples, 300 µL of Assay Buffer was transferred to the Non-Specific Binding (NSB) tubes, 200 µl of Assay Buffer to the Reference tubes, and 100 µl of Assay Buffer to each sample tube. 100 µL of Standards, Quality Controls, and Sample were transferred to appropriately labelled tubes in duplicate. 100 µL of hydrated ^{125}I-Insulin was transferred to all tubes followed by the addition of 100 µL of Human Insulin antibody to all tubes except the Total Count tubes and the NSB tubes. All tubes where than vortexed, covered and incubated overnight (~20-24 hours) at room temperature (22-25°C). After incubation 1.0 mL of cold (4°C) Precipitating Reagent was added to all tubes except the Total Count tubes, vortexed, and incubated for 20 minutes at 4°C. All tubes except the Total Count tubes were than centrifuged at 4°C for 40 minutes at 3000 x g. The supernatant was immediately decanted for all tubes except the Total Count tubes and drained for 60 seconds blotting excess liquid from the lip of the tube. Tubes where than counted in an automatic gamma counter (2470 WIZARD 2, PerkinElmer Life Sciences, Boston, MA) for 1 minute and raw data exported to a custom-made Microsoft Excel spreadsheet that adhered to the manufacturers guidelines for manual calculation of plasma insulin and are expressed in µU/mL. All Quality Control values measured were within the calculated acceptable Quality Control Range provided by the kit manufacturer.

Appendix E: Participant questionnaires and data sheets.

Recommended pre-trial diet for Chapters 3 and 4.

Diet: day before trial

24 Hour Diet Pre-Trial Day: Set Menu

No Exercise, Alcohol, Tobacco or Caffeine in the 48 hours before the trial!

BREAKFAST:

Cereal	90 g
Low-fat Milk	333 ml
4 slices of toast	*120 g*
Jam/Honey	*1 tablespoon*
1 tetra pack of orange juice	250 ml

LUNCH:

1 can of non caffeinated soft drink	375 ml
2 bread rolls	*180 g*
Half a tomato	*50 g*
Lettuce	*30 g*
Cheese	*40 g*
Ham (lean)	*60 g*
Margarine / Butter	*15 g*
1 apple	140 g

DINNER

Half a packet of pasta	175 g
Half a jar of pasta sauce	200 ml
1 can of non caffeinated soft drink	375 ml
2 "Two Fruits" snacks	280 g

SNACKS:

Please record any snacks eaten (type and amount) so that food intake is the same for both trials
Water may be consumed at any time during the day before the trial.

Possible snacks include:

1 banana	*140 g*
2 muesli bars	70 g
2 packets of sultanas	60 g
4 slices of bread with jam/ honey	*120 g and 1 tablespoon*

MORNING OF THE TRIAL:

Upon waking consume 5 ml of water per kilogram of body weight.
Eg. A 70kg person consumes 350 ml of water
An 83kg person consumes 415ml of water

Original Creators:
Matthew Watt and Mark Hargreaves.

Risk factor assessment questionnaire for Chapters 3, 4, and 5.

Risk factor assessment questionnaire

NAME: _____ **DATE** _____

M / F

ADDRESS: _____ **SEX**

_____ **Postcode:** _____ **AGE** _____ **YRS**

TELEPHONE: Work: _____ **WEIGHT** _____ **KG**

TELEPHONE: Mobile: _____ **HEIGHT** _____ **CM**

TELEPHONE: Home: _____ **EMAIL:** _____

MEDICAL HISTORY:

Medical Condition	NO	YES	DON'T KNOW	Medical Condition	NO	YES	DON'T KNOW
Heart Attack	❑	❑	n/a	Congenital Heart Disease	❑	❑	n/a
Chest Pain (angina)	❑	❑	❑	Disease of Arteries/Veins	❑	❑	❑
Heart Murmur	❑	❑	❑	Asthma	❑	❑	❑
Heart Rhythm Disturbance	❑	❑	❑	Lung Disease (eg. emphysema)	❑	❑	❑
Heart Valve Disease	❑	❑	❑	Epilepsy	❑	❑	❑
Heart Failure	❑	❑	❑	Stroke	❑	❑	n/a
*Back or neck injury	❑	❑	❑	*Shoulder, elbow or wrist injury	❑	❑	❑
* Bleeding disorder	❑	❑	❑	*Hip, knee or ankle injury	❑	❑	❑

***Give details of injuries to your back, neck, shoulders, elbows, wrists, hips, knees, or ankles in your medical history**

_____

_____

_____

List any prescribed medications being taken

_____

_____

_____

List any surgical procedures that you have had (write the year in brackets): example: appendix (1979)

_____

_____

_____

ALLERGIES: Do you have any allergies **NO** ❑ **YES** ❑ If yes, give details:

_____

SYMPTOMS DURING OR AFTER EXERCISE

As a result of exercise, have you ever experienced any of the following:

Symptom during exercise	NO	YES	Symptom during exercise	NO	YES
Pain or discomfort in the chest, back, arm, or jaw	❑	❑	Palpitations (heart rhythm disturbance)	❑	❑
Severe shortness of breath or problems with breathing during mild exertion	❑	❑	Pain in the legs during mild exertion	❑	❑
Dizziness, nausea or fainting	❑	❑	Severe heat exhaustion	❑	❑

CARDIOVASCULAR RISK FACTORS:

Do you have (tick NO, YES or circle ? for DON'T KNOW)

Cardiovascular Risk Factors	Yes	No	Don't know	
High Blood Pressure	❑	❑	?	
High Blood Cholesterol/Triglycerides	❑	❑	?	
Diabetes	❑	❑	?	
Current Smoker	❑	❑	Average/day =	
Ex-smoker	❑	❑	Average/day =	
Do you drink alcohol regularly?	❑	❑	Average/day=	drinks

FAMILY MEDICAL HISTORY:

Have members of your immediate family ever had any of the following conditions: (tick NO, YES or circle ? for DON'T KNOW). If you answer Yes or ?, write beside this the member of the family affected (F=father, M=mother, B=brother, S=sister, GM=grandmother, GF=grandfather).

Family medical history	NO	YES	?	FAMILY MEMBER	AGE (Years)	ALIVE NOW?
Heart Attack	❑	❑	?	_____	_____	_____
Sudden death (due to heart problems)	❑	❑	?	_____	_____	_____
Chest Pain (Angina)	❑	❑	?	_____	_____	_____
Stroke	❑	❑	?	_____	_____	_____
High Blood Pressure	❑	❑	?	_____	_____	_____
High Blood Cholesterol/Triglycerides	❑	❑	?	_____	_____	_____

Diabetes	❑	❑	?	_____	_____	_____
Bleeding disorder	❑	❑	?	_____	_____	_____

Exercise

List the sports, exercise or physically active hobbies (eg. gardening or playing with the kids) that you are **currently** engaged in:

Sport/Activity	**Day(s) of week** **Sa-Su-Mo-Tu-We-Th-Fr**	**Time of the day** **eg. 6 p.m.**	**Approximate duration** **eg. 30 minutes**
		TOTAL	

Rest/Recreation

How many hours sleep do you usually have? _____ hours/night

On average how much time do you spend each day on passive hobbies (i.e. watching TV) or just relaxing?

_____ minutes/hours per day.

Do you feel that you usually get enough restful sleep and time to relax? **Yes/No**

Client Declaration

I declare that the above information is to my knowledge true and correct, and that I have not omitted any information that is requested on this form.

SIGNED: _____

DATE: _____

Medical clearance form for Chapters 4 and 5.

This section should be completed by the medical practitioner.

If available, please provide copies of recent medical tests (i.e. ECG, blood test etc').

(11) MEDICAL PRACTITIONER'S SUMMARY:

(a) Comments (detail any significant abnormalities, reservations or precautions):

_____

_____

_____

_____

_____

(b) Recommendations:

The medical practitioner should underline and initial the appropriate clause:

(i) Fit to undergo maximal exercise test

(ii) Fit to undergo sub-maximal exercise test

(iii) Not fit to undergo any exercise test.

Signed: _____

Name: _____

Date: _____

Contact Telephone Number: (Wk) _____ (Mobile) _____

Continuous glucose monitor participant information sheet for Chapter 5.

Daily Care for your Continuous Glucose Monitor System

Today (Day 1)
After the 2 hour warm up needed for each new sensor, an alarm will sound asking you to 'Enter Meter BG'.

Before you can enter a calibration you have to clear the alarm by pressing ESC, then ACT.

Test your blood glucose and then enter the values into the monitor. ACT, ACT, up or down arrows – scroll to enter your BG, then ACT.

You need to calibrate three times at different time points throughout the day. This is to train the new sensor by giving it reference points to ensure an accurate reading.

Calibrations are more accurate when entered before meals or at least 1.5-2 hrs after a meal (when blood sugar levels should be back to baseline).

Tomorrow (Day 2) and following Day (Day 3)
The sensor requires calibration three times per day. These should be carried out at the following times:

- first thing when you wake up
- before eating lunch
- before going to bed (providing your last meal was 1.5-2 hours ago)

If no calibrations are performed for 12 hours, then an alarm will sound until you enter a calibration.

Day 4
Calibrate in the morning before breakfast. Return to the Victoria University for removal of the CGM device

If you experience any problems or have any questions please get in touch with a member of the research team or contact the Medtronics 24hr helpline.

Medtronics 24hr helpline:
1800 777 808

Medtronics Alerts/Error database:
http://www.medtronicdiabetes.com/support/alerts

Notes:

Meal Markers:

- **Mark a meal immediately prior to eating.**
- **In addition also write down the details of diet in the log sheet provided.**
- **Do not worry about what you put in for grams for meal consumption on the receiver (just use whatever default is selected), but instead please write down details of your meals in the log sheet provided.**

General:

- **The transmitter/sensor (the device attached to your skin) can store up to 40 minutes of data when away from the receiver (belt device), thus it is not necessary to carry the receiver with you for certain activities (e.g taking a shower).**
- **The receiver is not water proof (the belt device) but the sensor/transmitter (the device attached to your skin) is.**
- **The receiver can pick up the sensor/transmitter approximately 1 meter away so it may be better to place the receiver next to you on the bed when sleeping.**

Calibration Notes

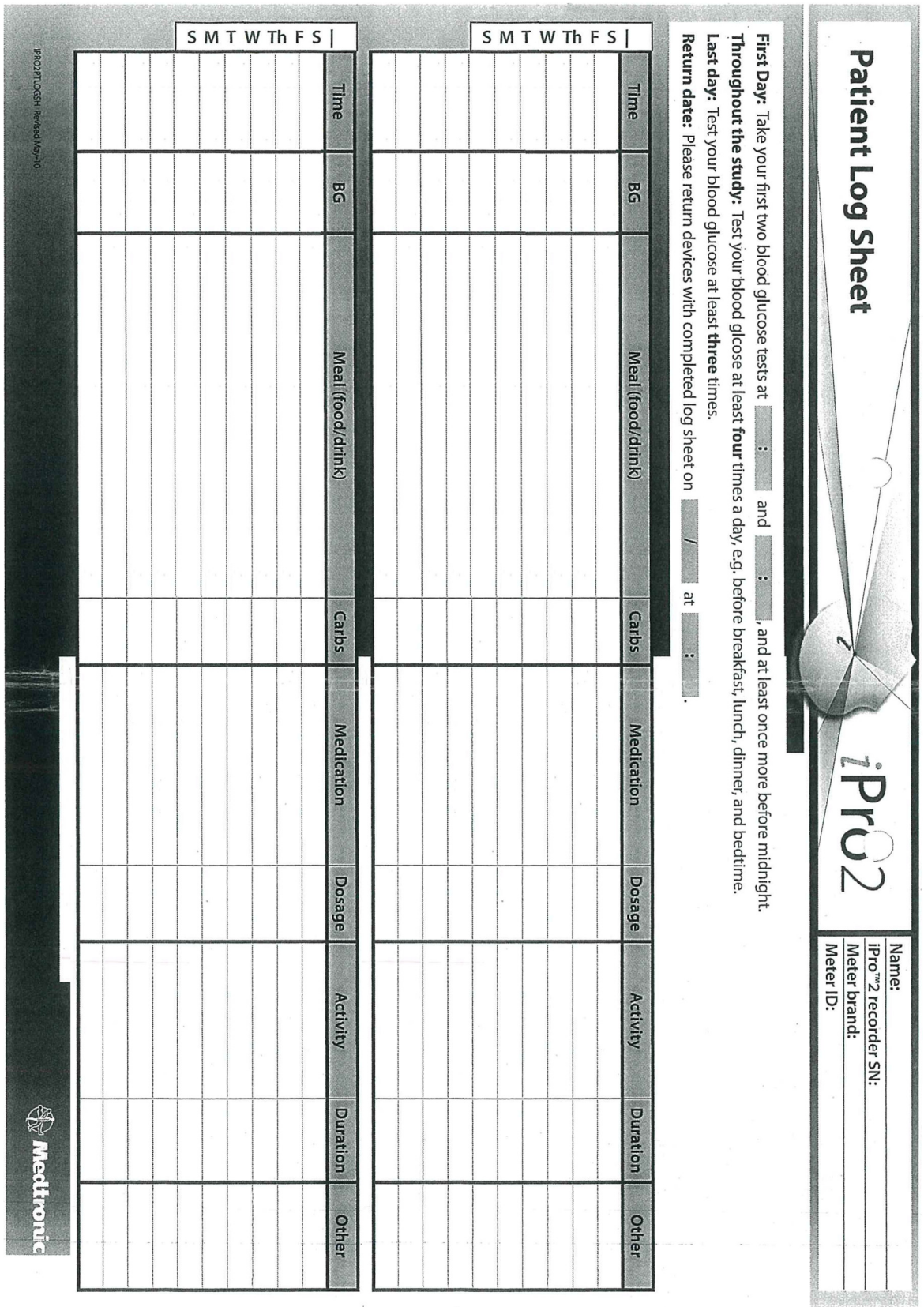
- **Calibration must be conducted in a fasted state (e.g no eating 2-3 hrs prior)**
- **You should calibrate the CGM 3 times per day**
    i. **prior to breakfast (immediately after waking up)**
    ii. **prior to lunch**
    iii. **prior to going to bed/sleep.**
- **To avoid contamination it is recommended that hands be washed in warm soapy water prior to puncturing the skin for blood glucose blood sample. Please ensure your <u>skin is dry</u> before taking the finger prick.**

Troubleshooting:

Lost sensor: Click Home Screen > Main Menu > Sensor > Sensor Start > Find Lost Sensor

End Sensor Life: Click Home Screen > Main Menu > Sensor > Sensor Start > New Sensor

Participant diet and physical activity diary for Chapter 5.



Patient Log Sheet

iPro2

Name:
iPro™2 recorder SN:
Meter brand:
Meter ID:

First Day: Take your first two blood glucose tests at : and : , and at least once more before midnight.
Throughout the study: Test your blood glcose at least **four** times a day, e.g. before breakfast, lunch, dinner, and bedtime.
Last day: Test your blood glucose at least **three** times.
Return date: Please return devices with completed log sheet on / at : .

S M T W Th F S

Time	BG	Meal (food/drink)	Carbs	Medication	Dosage	Activity	Duration	Other

S M T W Th F S

Time	BG	Meal (food/drink)	Carbs	Medication	Dosage	Activity	Duration	Other

IPRO2PTLOGSH Revised May-10

Medtronic

Rating of perceived exertion scale for Chapters 3, 4 and 5.

6	No exertion at all
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard (heavy)
16	
17	Very Hard
18	
19	Extremely hard
20	Maximal exertion

Borg (53)