

**Exploring changes in markers of oxidative stress and
inflammation in response to exercise**

by

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Abstract

Oxidative stress and inflammation are two reciprocally linked processes that characterise many disease states, but can also transiently increase in response to a range of stimuli, including exercise, to initiate adaptation. This thesis presents novel data indicating that oxidative stress did not increase in response to an acute mental arithmetic challenge, under resting conditions or experimentally induced high baseline inflammation. In the context of exercise, chapters 3, 4 and 5 support previous work that markers of plasma and peripheral blood mononuclear cell (PBMC) oxidative stress increase in response to acute exercise. Acute exercise (30-40 min, 70% $\text{VO}_{2\text{MAX}}$) in rheumatoid arthritis patients caused a transient increase in protein carbonylation that over a period of training (3 months, 3 sessions per week) at the same intensity did not increase global oxidative stress or inflammation. Plasma 3-nitrotyrosine decreased with exercise training, alongside improvements in disease activity and aerobic fitness. The last two experimental chapters of this thesis explored acute changes in plasma (Chapter 4) and PBMC (Chapter 5) oxidative stress in response to bouts of low volume high intensity interval training (LV-HIIT) and steady state exercise (60% maximal oxygen consumption ($\text{VO}_{2\text{MAX}}$), 27 minutes and 80% $\text{VO}_{2\text{MAX}}$, 20 minutes). LV-HIIT provided an anti-inflammatory (IL-6 and IL-10) and anti-oxidant (plasma total antioxidant capacity and PBMC thioredoxin protein content) response to exercise that paralleled the response (magnitude and timecourse) observed with steady state exercise of high and moderate intensity.

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To all now, the book is done...

This thesis is dedicated to the one true love of my life, Laura.

List of Publications

This thesis incorporates four papers, each corresponding to one of four experimental chapters:

1. Wadley, A.J., Veldhuijzen van Zanten, J.C.S., Paine, N.J., Drayson, M.T., & Aldred, S. (2013). Underlying inflammation has no impact on the oxidative stress response to acute mental stress, *Brain, Behaviour and Immunity* (In Press).
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Other publications:

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2. Paine, N.J., Ring, C., Aldred, A., Bosch, J.A., Wadley, A.J., & Veldhuijzen van Zanten, J.C.S. (2013). Eccentric-exercise induced inflammation attenuates the vascular responses to mental stress. *Brain, Behaviour and Immunity*, 30, 133-142.
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List of Abbreviations

AAPH	azo-bis dihydrochloride
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BP	Blood pressure
BMI	Body mass index
BSA	Bovine serum albumin
CRP	C-reactive protein
ddH ₂ O	Double distilled water
DAS28	Disease activity score 28
DBP	Diastolic blood pressure
DMARDs	Disease-modifying antirheumatic drugs
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DNPH	Dinitrophenylhydrazine
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme linked immunosorbent assay
ETT	Exercise tolerance test
FRAP	Ferric reducing ability of plasma
GPx	Glutathione peroxidase
GSH	Reduced glutathione
HDL	High density lipoprotein
HR	Heart rate
H ₂ SO ₄	Sulphuric acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgE	Immunoglobulin E
IL	Interleukin
IM	Intra-muscular
IV	Intra-venous
LDL	Low density lipoprotein
LOOH	Lipid hydroperoxide
mA	milliamp

mg	milligram
ml	milli litre
mmol	millimole
MnSOD	Manganese Superoxide Dismutase
n	number of participants
nmol	nanomole
NaCl	Sodium chloride
NED	N-(1-naphthyl) ethylenediamine dihydrochloride
NO _x	Nitric oxide metabolites
3-NT	3-Nitrotyrosine
NUNC	Trademark of Nalge Nunc International
OPD	O-phenylenediamine
OSA	Octanesulfonic acid
Ox-LDL	Oxidised low density lipoprotein
PAGE	Polyacrylamide gel electrophoresis
PC	Protein carbonyl
PRDX	Peroxiredoxin
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species

RONS	Reactive nitrogen species
RPE	Rating of perceived exertion
RPM	Revolutions per minute
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard Error
SDS	Sodium dodecyl sulfate
SPSS	Trademark of SPSS Inc.
TAC	Total antioxidant capacity
TBS	Tris buffed saline
TBST	Tris buffed saline with Tween 20
TCA	Trichloroacetic acid
TLR	Toll-like receptor
TPZ	Tris (2-pyridyl)-S-triazine
TRX	Thioredoxin
TRX-R	Thioredoxin reductase
TXIP	Thioredoxin interacting protein
V	Voltage
VO _{2MAX}	Maximal oxygen consumption

Chapter One

1. General Introduction

1.1. Summary

Oxidative stress (Harman, 2006; Seven et al., 2008) and inflammation (Singh and Newman, 2010; Libby, 2008) are two processes that impact upon health, fitness, ageing and disease. Acute increases in exercise-induced oxidative stress and inflammation have been linked with post-exercise adaptations (Gomez-Cabrera et al., 2008; Gleeson et al., 2011). Furthermore, evidence suggests a reciprocal relationship between oxidative stress and inflammation, whereby each process may initiate and progress the other (Wadley et al., 2013). The aims of this thesis were to assess oxidative stress and inflammation in response to various stimuli, with a primary focus on exploring and characterising the responses to exercise. Studies were designed to investigate potential links between oxidative stress and inflammation (Zhou et al., 2010; Riddell et al., 2010; Fu et al., 2002), mostly in the context of exercise.

1.2. Oxidative Stress

1.2.1. Overview

Reactive Oxygen and Nitrogen species (ROS and RNS, collectively RONS) are highly reactive natural derivatives of cellular oxidation processes, that are almost always characterised by the presence of one or more unpaired electrons in their outer shells (i.e. free radical species). RONS are produced from an array of environmental (i.e. radiation and inorganic particles) (Martin et al., 1996) and biological (endogenous oxidase enzymes, electron transport chain and peroxisomes) sources (Brandes et al., 2010) and are key in the regulation of normal biological processes such as cellular respiration and signalling (Apel and Hirt, 2004). The reduction of molecular oxygen to form superoxide ($O_2^{\cdot-}$) is central (directly

or indirectly) in the formation of other RONS such as peroxynitrite (ONOO^-), lipid peroxy (LOO^\cdot) and hydroxyl radicals (OH^\cdot).

Antioxidants are a counterbalance to the reactivity of RONS that function by directly ‘quenching’ RONS or indirectly, by reducing oxidised substrates (Nordberg and Arner, 2001) and stimulating the transcription of other antioxidant systems (Burke-Gaffney et al., 2005). In situations where antioxidant defences can effectively regulate the levels of RONS, the net effect is the promotion of adaptive physiological responses, such as increased expression of protective antioxidant enzymes and stress proteins (Ji, 2001). This response helps to maintain redox balance and preserve free cellular thiols (Jacob and Ba, 2011). Conversely, a substantial pro-oxidant shift in the balance between RONS and antioxidants can facilitate adduct formation on proteins, lipids and DNA. This situation is termed ‘oxidative stress’ and is associated with the ageing process (Harman, 1956) and many disease pathologies (Seven et al., 2008; Butterfield et al., 2006; Dhalla et al., 2000; Ceriello and Motz, 2004).

Due to the transient nature of RONS, it is common practice to measure the formation of adducts (Harman, 2006), which may act as ‘‘footprints’’ of RONS-mediated reactions. Care must be taken when interpreting results employing these methods, as markers of oxidative stress are not direct measures of RONS production. However, markers of biomolecule oxidation/nitration are routinely measured in biomedical research and are widely accepted as reflective markers of oxidative stress.

1.2.2. Protein Oxidation

The backbone of a protein polypeptide chain is highly susceptible to reaction with RONS (Stadtman and Berlett, 1997). Certain amino acids (i.e. histidine, arginine and lysine) contain redox active sulphur-hydryl ($-\text{SH}$) groups, also called thiol groups that similarly to

some RONS contain unpaired outer shell electrons. As a result, during heightened periods of oxidative stress, amino acid side chains can be targeted by RONS such as OH^\cdot , causing oxidative modifications that stabilise outer shell stability of both molecules. Mildly oxidised proteins can be reduced by antioxidant systems or degraded and removed by the proteasome system (Breusing et al., 2009), however excess protein oxidation can lead to protein aggregation and inhibition of cellular and non-cellular processes (Goto et al., 2007). Common protein adducts include dityrosine, protein-protein cross linking and protein carbonyl groups. Protein carbonyls are the most studied marker of protein oxidation *in vivo* (Dalle-Donne et al., 2003; Levine et al., 1994), due to their immunogenic properties and ability to derivatize with 2, 4-dinitrophenylhydrazine (DNPH) to form a 2,4-dinitrophenyl group (Reznick and Packer, 1994). This can be easily detected using spectrophotometric assays (Carty et al., 2000) or antibody technology (i.e. Enzyme-linked immunosorbent assay (ELISA) (Buss et al., 1997) or western blotting (Levine et al., 1994)).

1.2.3. Protein Nitration

Protein nitration occurs as part of RNS-mediated signal transduction by secondary products of nitric oxide (NO) metabolism, such as peroxynitrite (ONOO^-) and nitrogen dioxide (NO_2^\cdot). ONOO^- is formed from the reaction between NO and O_2^\cdot (Beckman, 1996) and outcompetes the removal of O_2^\cdot by superoxide dismutase (SD), thus making ONOO^- a highly prominent radical species (Beckman et al., 1990). ONOO^- can nitrate amino acids, commonly tyrosine, by adding a nitro group to the benzene ring that forms the adduct 3-nitrotyrosine (3-NT) (Souza et al., 2008). This conformational change to cellular (Benhar et al., 2008, 2010) and non-cellular proteins (Griffiths et al., 2006) can regulate various aspects of protein function (Benhar et al., 2008). Examples include tyrosine phosphorylation signalling (Kong et al., 1996) and lipoprotein regulation (Griffiths et al., 2006) respectively.

3-NT is commonly quantified using high performance liquid chromatography with electrochemical detection (EC-HPLC), however ELISA and western blotting are also used (Herce-Pagliai et al., 1998).

1.2.4. Lipid Oxidation

Cellular and non-cellular polyunsaturated fatty acids (PUFA) are susceptible to reaction with RONS (Niki, 2009). Double bonds in PUFA's (i.e. arachidonic and linoleic acids) of phospholipid membranes and plasma lipids (i.e. fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, and prenols (Quehenberger et al., 2010)) and low density lipoproteins (LDL) can be targeted by RONS forming stable lipid peroxidation (LPO) end products. The abstraction of hydrogen from the methylene group of PUFA's can form a carbon radical that can rearrange to form conjugated dienes, that can be further oxidised to form lipid and peroxy radicals (Buettner, 1993). These highly reactive radical species can then propagate a chain reaction whereby multiple LPO products are formed, including lipid hydroperoxides (LOOH) and short (malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE)) and long chain (i.e. F2-isoprostanes) aldehyde products (Niki, 2009; Buettner, 1993). Increased formation of LPO products can modulate systemic lipid metabolism (Fuhrman et al., 1995) and the assembly of cell membranes, altering fluidity, permeability and functioning of cellular signalling (Nikolaidis et al., 2011). Quantification of LPO products can be achieved through antibody technology (i.e. 4-HNE), mass spectrometry (i.e. F2-isoprostanes) and spectrophotometric assays (i.e. LOOH and MDA) (Breusing et al., 2010).

1.2.5. Antioxidants

Indicators of antioxidant status primarily differ by the function (enzymatic and non-enzymatic), target (i.e. specific RONS or oxidised molecule), location (i.e. endogenous and exogenous) and routine method of quantification (i.e. protein expression, gene expression or enzymatic activity) of the antioxidant. Many antioxidants function by mimicking the downstream target of a specific RONS, or by reversing/suppressing specific oxidation events on proteins, lipids or DNA molecules. An example is the binding of antioxidants to proteins and DNA to suppress iron-mediated oxidation (Wiedenheft et al., 2005). Common measures of antioxidant capacity include the assessment of the protein expression and activity of reduced:oxidised glutathione (GSH:GSSG ratio) and RONS specific antioxidant enzymes (SOD and catalase (CAT)). In addition, plasma contains a multitude of soluble antioxidant compounds such as vitamins (ascorbic acid and α -tocopherol), albumin bound thiols, bilirubin and uric acid that can be collectively assessed using total antioxidant capacity (TAC) assays (Cao and Prior, 1998). Finally, the assessment of peroxide scavenging proteins thioredoxin (TRX) and peroxiredoxin (PRDX) are emerging as indicators of cellular antioxidant status.

1.3. Inflammation

1.3.1. Overview

Inflammation is a bodily defence mechanism that is initiated in response to tissue damage or infection, mediated by signalling cytokine molecules, and characterised by the influx of migrating leukocytes (Medzhitov, 2008). Acute inflammation is a transient, adaptive response leading to transcriptional activation of multiple anti-inflammatory cytokines, whereas chronic inflammation is characterised by an “overdrive” of inflammatory mediators,

progressing to increased levels of systemic inflammation (Stevens et al., 2005). Increased levels of systemic inflammation are implicated in many disease states, notably dementia (Engelhart et al., 2004), rheumatoid arthritis (Lee and Weinblatt, 2001), and cardiovascular disease (Tracy, 1998). Indeed C-reactive protein (CRP), a marker of systemic inflammation, is present within most atherosclerotic plaques and is implicated in the pathogenesis, progression and complications of cardiovascular disease (Devaraj et al., 2009).

The inflammatory response (*figure 1.1*) is initiated by a rapid infiltration of leukocytes to the site of infection, which then engulf the invading pathogen. During this response neutrophils weakly adhere to the blood vessel wall via cellular adhesion molecules (CAMs), that can initiate a rolling motion (Mayrovitz et al., 1977). The release of neutrophils to the site of infection is then mediated by a chemotactic gradient across the endothelial wall. Interleukin (IL)-1 β has been identified as the chemokine involved in this initial inflammatory process, alongside IL-8, which directly recruits neutrophils to the site of tissue damage (Utgaard et al., 1998). Another cytokine, IL-6, then recruits monocytes into blood vessels, instigating the differentiation of monocytes into pathogen-disposing macrophages (Kaplanski et al., 2003). Additionally, Tumour Necrosis Factor-alpha (TNF- α) is involved in the recruitment of CAMs to the endothelial wall (Willerson and Ridker, 2004; Bruunsgaard, 2005).

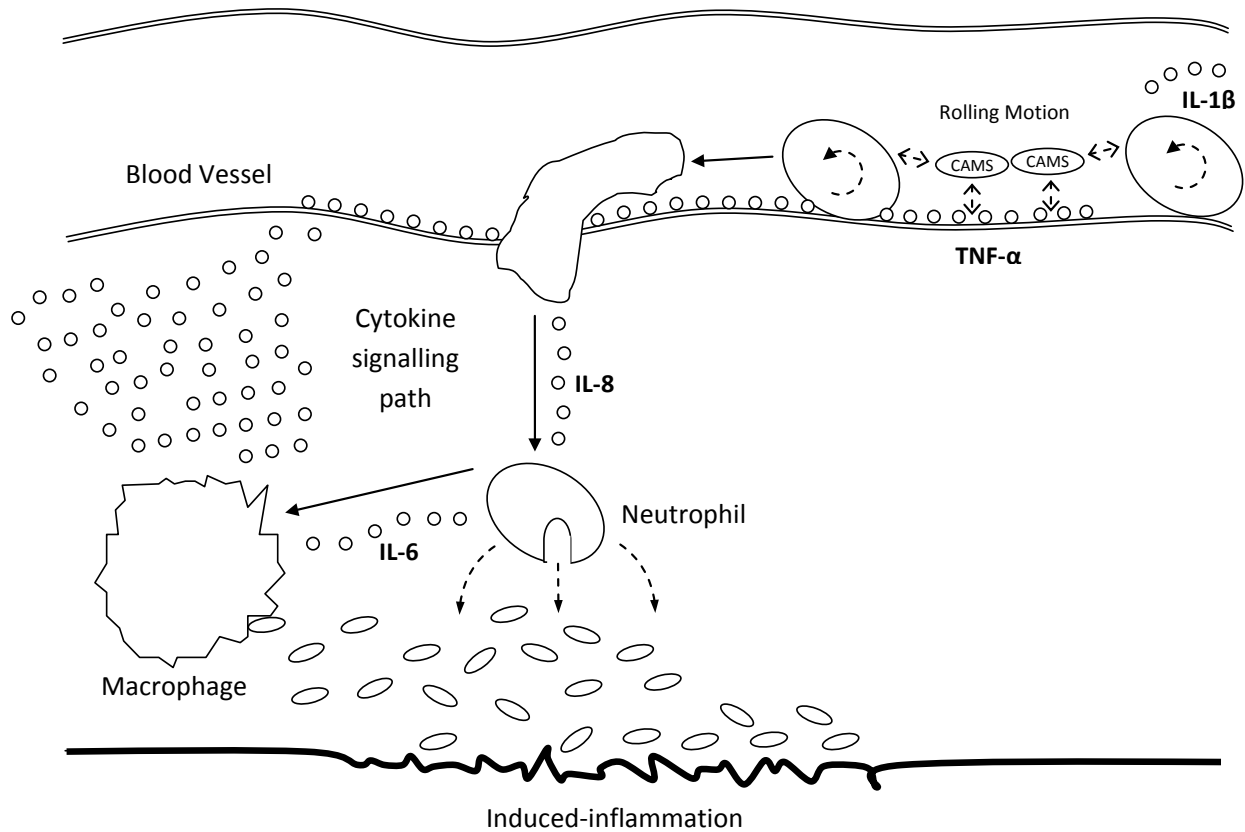


Figure 1.1: Typical Inflammatory Response. Blood leukocytes can travel to the site of tissue damage through the actions of cytokine molecules that mediate cellular recruitment (IL-1 β , IL-8) adhesion (TNF- α), and differentiation (IL-6). These cytokine mediators allow specific leukocyte cell types to dispose of the inflammatory stimulus. CAMS = cellular adhesion molecule, ° denotes cytokine molecule (specific cytokines are indicated in bold where appropriate). All cell types in the above example are neutrophils unless stated. Figure adapted from Wadley et al, 2012, American Association of Aging.

1.3.2. Markers of Inflammation

The inflammatory response is characterised by a large shift in the production, mobilisation and distribution of leukocytes to an ‘injured’ area of the body (i.e. pathogen, tissue damage or pollutant). Shifts in the subsets of leukocytes can be measured using automated cell counters (Cox et al., 1985) or microscopy using Wright’s reagent staining

(Stain et al., 2013). Cytokine molecules are small polypeptides released either locally (autocrine and paracrine) or systemically (endocrine) from leukocytes to mobilise further leukocyte sub sets as well as signalling to cellular kinases to regulate the activity and transcription of adaptive proteins (Febbraio et al., 2010). Many cytokines facilitate the inflammatory process and the relevance for each marker to the separate studies will be detailed in each chapter. Briefly, interleukin (IL-) 1 β , IL-8, IL-6 and TNF- α cytokines are involved in the acute phase response (Gabay and Kushner, 1999) and are easily measured using sandwich ELISA techniques. Additionally, cytokines are known to stimulate the release of acute phase proteins such as CRP, serum amyloid A, complement proteins and fibrinogen from the liver (Stevens et al., 2005; Gabay and Kushner, 1999). These can all be measured as indicators of systemic inflammation.

1.4. Nitric Oxide

Nitric oxide (NO) is a radical species with diverse biological properties. NO Synthase (NOS) is the key regulatory enzyme in the synthesis of NO, comprising endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) isoforms. Further, recent evidence has demonstrated that NO can be synthesised through dietary nitrate (NO₃⁻) intake (Benjamin et al, 1994). Anaerobic bacteria in the enterosalivary circulation can reduce dietary NO₃⁻ to nitrite (NO₂⁻), which can be absorbed into the systemic circulation and reduced by various enzymes (xanthine oxidase and carbonic anhydrase) and reducing agents (ascorbate and polyphenols) to form NO under hypoxic conditions (Bailey et al, 2012). In small amounts NO can govern cellular growth and proliferation (Guzik et al., 2003) through regulation of cellular kinase activity, immune function and blood flow distribution via the dilation of smooth muscle cells (Lundberg and Weitzberg, 2009). Conversely, inflammation driven NO production is typically a much larger and sustained response that far surpasses the physiological amounts

required to govern NO related signalling and adaptation (Kim et al., 1999). As a result, NO produced via iNOS is typically linked with cellular arrest and apoptosis (Parratt, 1997).

NO is a transient molecule, due to its rapid oxidation *in vivo* (Beckman et al., 1990) and is therefore very difficult to directly monitor in blood or urine. A number of different measures have been used to try to assess NO availability. Traditional indirect methods involve functional assessments of NO-related vasomotion in response to physiological and/or pharmacological stimuli that block the actions of NO-mediated blood vessel dilation to test NO availability. Additionally, circulatory markers of NO metabolism, nitrite (NO_2^-) and nitrate (NO_3^-) are routinely assessed in plasma and urine as indicators of NO availability (Moshage et al., 1995). Given that dietary nitrate can largely influence NO metabolism and thus plasma NO_2^- and NO_3^- , strict dietary control is typically implemented in these studies. Quantification of NO_2^- can be achieved using a luminescent assay based technique (Moshage et al., 1995).

1.5. Oxidative Stress & Inflammation – Interactions and Similarities

1.5.1. Overview

Oxidative stress and pro-inflammatory processes are often deemed to be mutually dependent, with studies suggesting that oxidative stress is a direct stimulus for inflammation and vice versa (Kim et al., 2006). It has been established *in vitro* that neutrophils present during an inflammatory response may directly produce ROS, such as O_2^- (Babior et al., 1973). In addition, RONS (Gloire et al., 2006) and inflammatory cytokines (Donato et al., 2009) have both been shown to stimulate the redox-sensitive transcription factor NF- κ B, and although NF- κ B is a relatively non-specific transcription factor this does link oxidative stress and inflammation with a common integrative source (*figure 1.2*). Furthermore, the oxidation

of mitogen activated protein kinase (MAPK) (i.e. kinase/phosphatase balance) (Naik and Dixit, 2011; Choi et al., 2002), an upstream regulator of NF- κ B, may directly and indirectly modulate inflammation respectively. Despite these documented links, novel protein markers appear to provide further upstream links between oxidative stress and inflammation. Inflammatory proteins such as α -1-antitrypsin, α -1-proteinase inhibitor, α -1-antiproteinase (Choi et al., 2002) and complement protein 4 (Hart et al., 2004) are all susceptible to oxidation, which may enhance inflammation (Kalsheker, 1994; Moraga, 2000). The roles of IL-8 and the redox proteins TRX and PRDX are primary focus of this thesis and may provide mechanistic links between oxidative stress and inflammation.

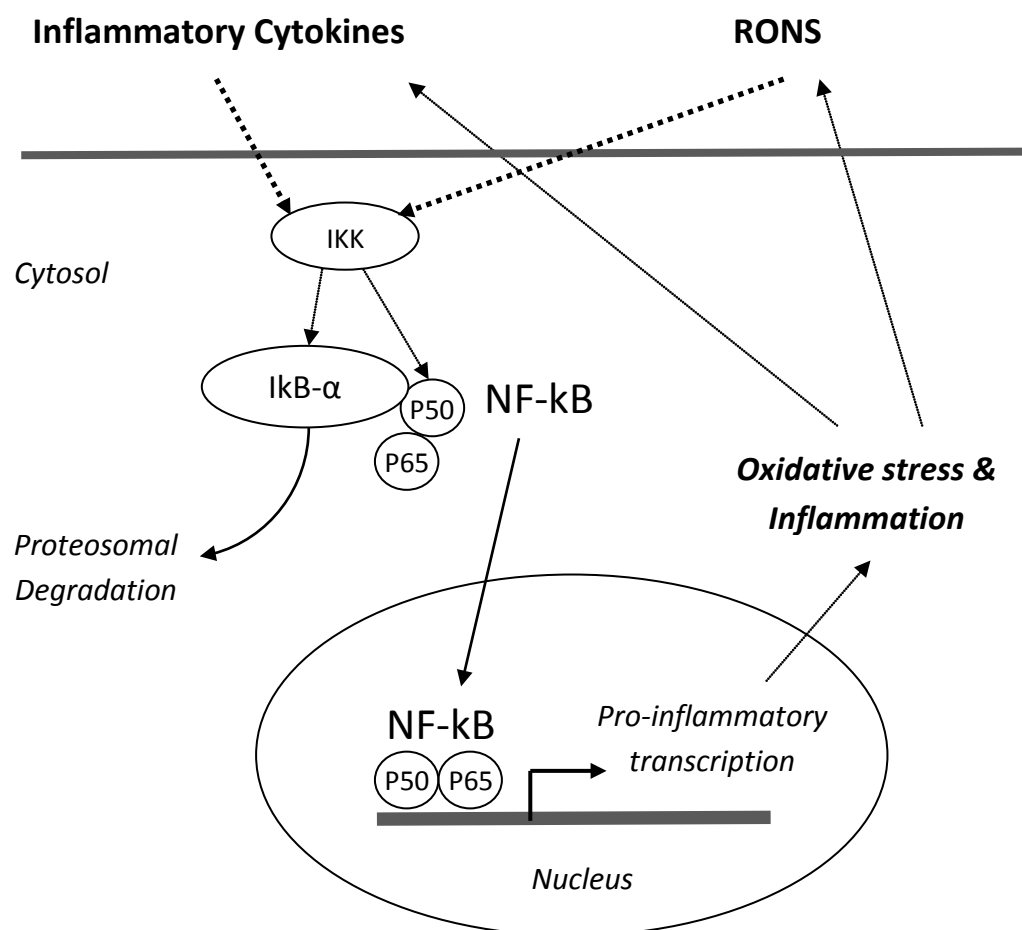


Figure 1.2: Activation of NF- κ B by reactive oxygen species and inflammatory cytokines. NF- κ B is constitutively expressed in the cytosol of cells, bound to inhibitory-

kappa B Kinase (IKK). RONS and inflammatory cytokines can activate IKK to preferentially phosphorylate specific sub-units of NF- κ B to initiate migration into the nucleus for pro-inflammatory transcription.

1.5.2. Interleukin-8 as a link between oxidative stress and inflammation

IL-8 has been suggested to be a link between oxidative stress and inflammation, via the metabolism of prostaglandins. Prostaglandins are compounds derived from fatty acids that regulate cellular growth and differentiation near the site of their release (Smith, 1989). It has been proposed that 15-Deoxy-Delta-12,14-prostaglandin J2 may directly modify components of the electron transport chain and potentiate radical leakage (Kondo et al., 2001). Evidence suggests that radicals may then signal via MAPK to directly upregulate the expression of IL-8 cytokines (Fu et al., 2002). IL-8 is a cytokine known to recruit neutrophils at the beginning of the inflammatory response (Utgaard et al., 1998), which may initiate a respiratory burst (Babior et al., 1973), thus providing a full cyclic link between IL-8 and oxidative stress.

1.5.3. Thioredoxin

TRX is a ubiquitous oxidoreductase protein present within the cytoplasm, nuclei and mitochondria of cells (Go et al., 2007). TRX can exist in a reduced or oxidised state, dependent on the level of systemic oxidative stress and availability of redox sensitive thiol groups. When in a reduced state, TRX can act as an antioxidant, utilising its thiol group to directly scavenge ROS, as well as activating the expression of various antioxidant enzymes (Burke-Gaffney et al., 2005) and regenerating GSH (Tan et al., 2010) and vitamin C (May et al., 1997). In its oxidised form, TRX forms an intra-molecular disulphide bridge that blocks antioxidant function. Recent evidence has implicated a TRX binding protein, Thioredoxin-

interacting protein (TXNIP), as a link between elevated oxidative stress and inflammation (Zhou et al., 2010; World et al., 2011; Lane et al., 2013) (figure 1.3). TXNIP can bind to TRX via a disulphide bond, inhibiting TRX antioxidant function (Patwari et al., 2006). An increase in ROS has been shown to promote dissociation of TXNIP from TRX, enabling TRX to scavenge ROS, whilst TXNIP activates the NLP3 inflammasome to produce the inflammatory cytokine, IL-1 β (Zhou et al., 2010). IL-1 β is implicated with the activation of various other cytokines and evidence has drawn associations between TRX-TXNIP with IL-1 α , IL-6, IL-8, TNF- α and IL-2 production (Yamada et al., 2003; Schenk et al., 1996). It therefore appears that despite the antioxidant properties of TRX, TXNIP can indirectly enhance the inflammatory signal during periods of heightened oxidative stress. Indeed, there is some evidence implicating elevated TXNIP protein expression with metabolic (Zhou et al., 2010) and cardiovascular (Schulze et al., 2006) pathology.

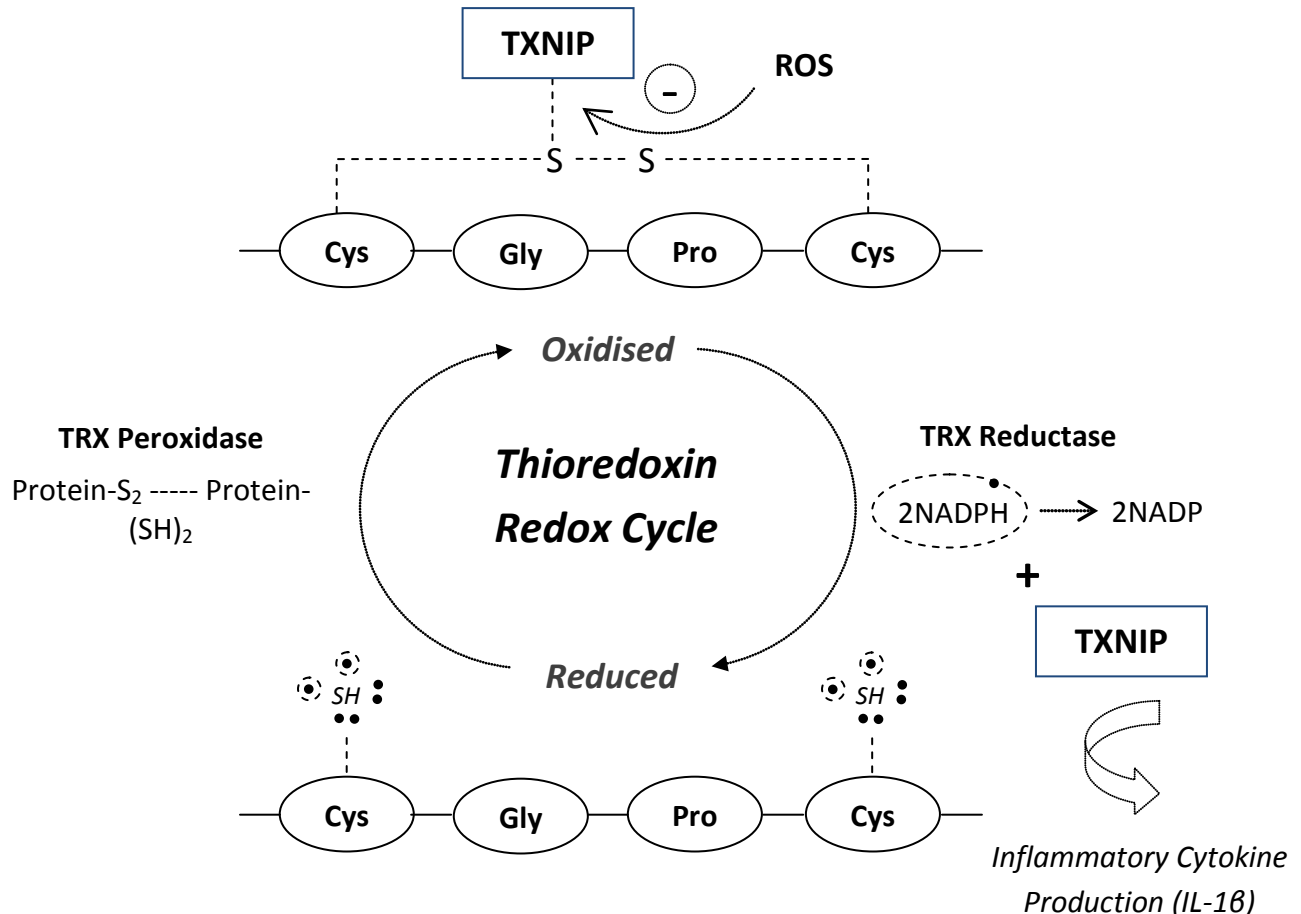


Figure 1.3: Schematic of the TRX redox cycle. TXNIP can form a disulphide bond with the active site of TRX, blocking TRX antioxidant function. Unbound TXNIP is associated with heightened inflammatory cytokine production.

1.5.4. Peroxiredoxin

PRDX are a family of oxidoreductase proteins containing thiol groups with a high capacity to control cellular levels of RONS and reduce oxidative stress (Schenk et al., 1994; Wood et al., 2003). PRDX can exist in a series of oxidation states that allows the enzyme to directly target and reduce biological peroxides such as hydrogen peroxide (H_2O_2), ONOO^- and LOOH (Wood et al., 2003) (figure 1.4). Further, this allows PRDX to regulate peroxide mediated-signalling, primarily within lipid rafts of cells (Rhee et al., 2012). Recent work has highlighted the role of PRDX in modulating inflammation and immune function (Riddell et al., 2010). PRDX appears to have intra and extra-cellular functions that may protect against infection, cell death and cancer (Ishii et al., 2012). Work in isolated macrophages and dendritic cells has indicated that PRDX can bind to toll-like receptor-4 (TLR-4) and stimulate the production of inflammatory cytokines such as $\text{TNF-}\alpha$ and IL-6, via NF- κB (Riddell et al., 2010). It therefore appears that PRDX may provide a novel link between oxidative stress and inflammatory processes. Additionally, TRX reductase appears to provide a link between TRX and PRDX redox protein systems by directly and indirectly facilitating reformation of the reduced forms of both proteins (figure 1.4).

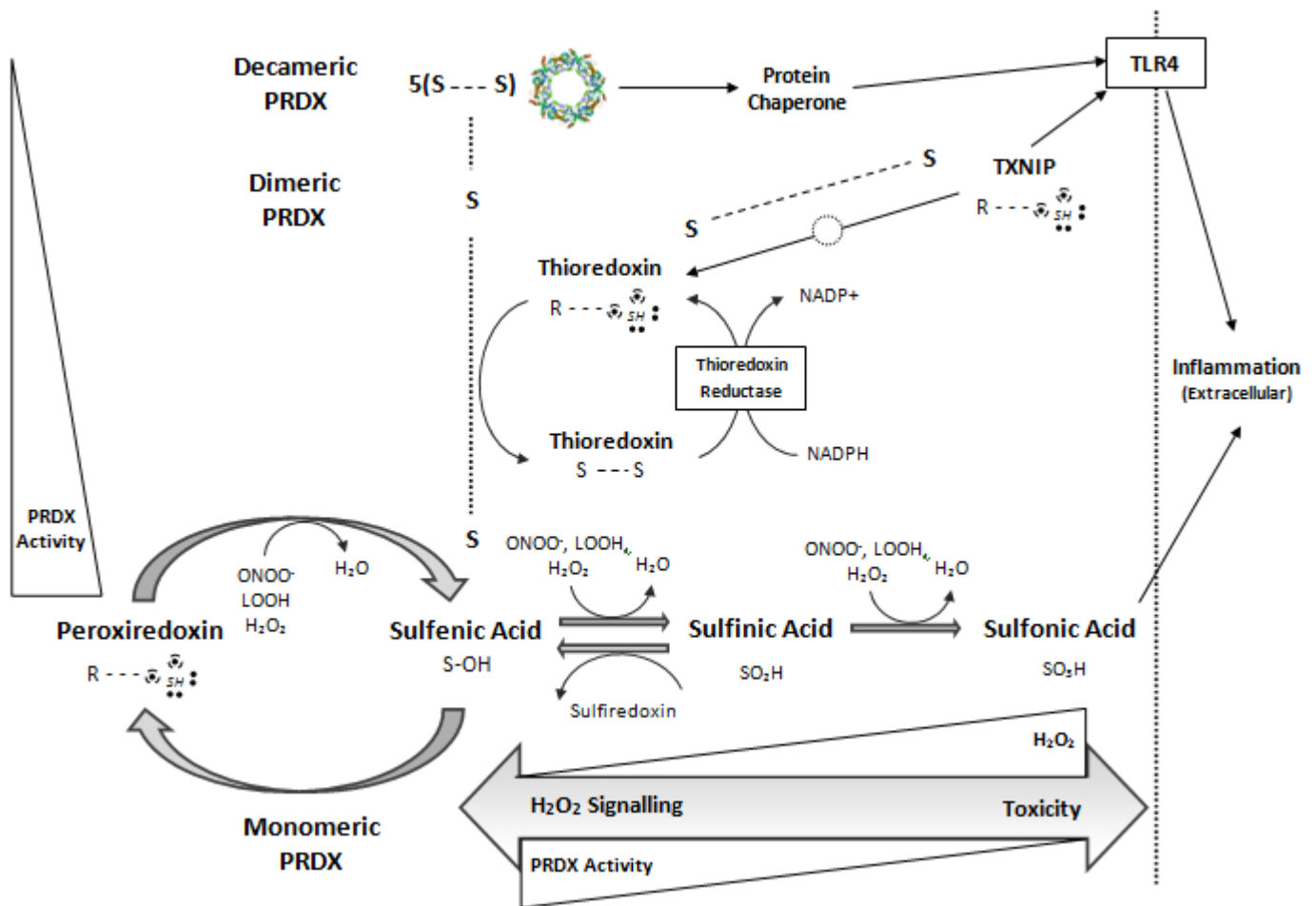


Figure 1.4: Schematic of the PRDX redox cycle. Schematic indicates how TRX can regulate PRDX oxidation by reversing PRDX thiol oxidation. Under conditions of high or prolonged peroxide exposure, PRDX can exceed the regulatory control of TRX and become over-oxidised. Over-oxidation of PRDX has been associated with heightened inflammation via toll like receptor (TLR) activation. Previously undefined terms: S - - - S denotes a disulphide bond. R represents an R group.

1.5.5. Summary

It may be possible that IL-8, TRX and PRDX contribute towards amplifying the inflammatory signal under conditions where oxidative stress is enhanced, rather than RONS inducing inflammation directly. Additionally, oxidative stress induced damage to cellular biomolecules such as proteins, lipids and DNA may well initiate inflammation in response to damage (Ungvari et al., 2010). However due to a nature of these studies in predominantly cell models, caution must be taken when applying these mechanisms in humans, given their complexity *in vivo*.

1.6. Oxidative Stress & Inflammation in Disease

Various genetic and environmental factors can contribute towards the development of human disease. A range of low (Yudkin, 2007; Gleeson et al., 2011) and high grade (Libby, 2008) inflammatory diseases such as cardiovascular disease (CVD) and rheumatoid arthritis (RA) respectively are characterised by increased levels of inflammatory proteins (IL-1 β , TNF- α , IL-6 and CRP). Further, CVD (Dhalla et al., 2000; Ceriello and Motz, 2004; Cai and Harrison, 2000) and RA (Seven et al., 2008; Taysi et al., 2002; Sarban et al., 2005) are associated with elevated levels of oxidative stress. The deleterious effects of oxidative stress and inflammation in human disease are specific to each disease state, but commonly involve degradation of tissue structure or function via excessive oxidative modification (Mapp et al., 1995). This may lead to activation of apoptotic pathways or conversely an inhibition, resulting in a lack of cell clearance (Szabó-Taylor et al., 2012) which can lead to their aggregation and ultimately impaired tissue functionality. For example in CVD this may manifest as impaired cardiac cell function (Bains and Shaw, 1997; Butterfield et al., 2006) and in RA, the degradation of connective tissue in a joint (Mapp et al., 1995). Interestingly, both of these diseases are linked by vascular complications and associations with altered NO

metabolism (Griffiths et al., 2006; Metsios et al., 2010; Bennett et al., 2009). This will be an underlying theme to the first two experimental chapters of this thesis. Evidence suggests that oxidative stress and inflammatory processes seen in human disease may disrupt the chain of NO synthesis and function, via ONOO⁻ formation (Pryor and Squadrito, 1995; Beckman and Koppenol, 1996), which directly attenuates NO-mediated function. Conversely, high levels of inflammation NO production can exacerbate oxidative stress and act toxic to cells (Kim et al., 1999). To understand the complex roles of NO in health and disease, parallel measures of oxidative stress and inflammation must be considered.

1.7. Changes in markers of oxidative stress and inflammation during and following exercise

1.7.1. Overview

Exercise is beneficial for health, and is known to improve cardiovascular function, physical fitness and psychosocial health (Metsios et al., 2008). Regular exercise is associated with a reduction in multiple cardiovascular risk factors such as correction lipoprotein profiles, lowered fat mass and blood pressure (Shephard and Balady, 1999) as well as improved vascular function (Clarkson et al., 1999) and enhanced NO release (Lewis et al., 1999). Additionally, there is evidence that systemic inflammation (Colbert et al., 2004; Dekker et al., 2007; Adamopoulos et al., 2002) and oxidative stress (Donato et al., 2010; Radak et al., 1999; Fatouros et al., 2004) are both independently reduced as a result of regular physical exercise.

1.7.2. Acute increases in oxidative stress during and following exercise

It is now widely accepted that a single bout of exercise is accompanied by a transient increase in markers of oxidative stress (Fisher-Wellman and Bloomer, 2009; Michailidis et al.,

2007). Studies examining the responses to exercise have reported that markers of oxidative stress positively correlate with muscle damage (Aoi et al., 2004). However, studies utilising antioxidant supplementation have demonstrated that quenching radical release in response to exercise can prevent not only muscle damage (Dekkers et al., 1996; Goldfarb et al., 1994), but a diverse range of signalling events that ultimately lead to adaptation (Ristow et al., 2009; Gomez-Cabrera et al., 2008; Khassaf et al., 2003; Hart et al., 2013). Early studies identified that RONS have significant roles in stimulating muscle force production (Andrade et al., 1998), enzyme release (Jackson et al., 1983, 2004) and the transcriptional regulation of critical cellular proteins, such as heat shock proteins (Salo et al., 1991) and antioxidant enzymes (Khassaf et al., 2003). In addition, recent findings have reported that RONS may regulate improvements in insulin sensitivity (Ristow et al., 2009), mitochondrial biogenesis (Ristow et al., 2009) and endurance capacity (Gomez-Cabrera et al., 2008). Interestingly, antioxidant supplementation has also been shown to suppress the anti-inflammatory response to exercise, by reducing muscle derived IL-6 production (Fischer et al., 2004; Vassilakopoulos et al., 2003). This underpins the links between oxidative stress and inflammation, specifically in the context of exercise.

1.7.3. Measuring exercise-induced oxidative stress: beyond the muscle

It is well established that activated skeletal muscle is a prominent source of RONS during and following exercise (McArdle et al., 2001, 2005; Davies et al., 1982) (figure 1.5). $O_2^{\cdot -}$ is produced from a range cellular sources within skeletal muscle, notably the mitochondria, peroxisomes and via enzymes such as nicotinamide adenine dinucleotide (NAD) oxidases, xanthine oxidase, eNOS and nNOS (Powers and Jackson, 2008; Jackson, 2005). Muscle cells can also release radicals into the extracellular space via enzymes expressed on the plasma membrane of the cell (McArdle et al., 2001; Balon and Nadler, 1994;

O'Neill et al., 1996). Furthermore, radicals may be produced by cells surrounding and infiltrating skeletal muscle, such as endothelial cells (Li and Shah, 2004) and many subsets of leukocytes (Babior, 2000) in a controlled and regulated manner (Jackson, 2008). As a result markers of exercise-induced oxidative stress are routinely measured in the systemic circulation (Goldfarb et al., 2005; Bloomer et al., 2005; Turner et al., 2011b).

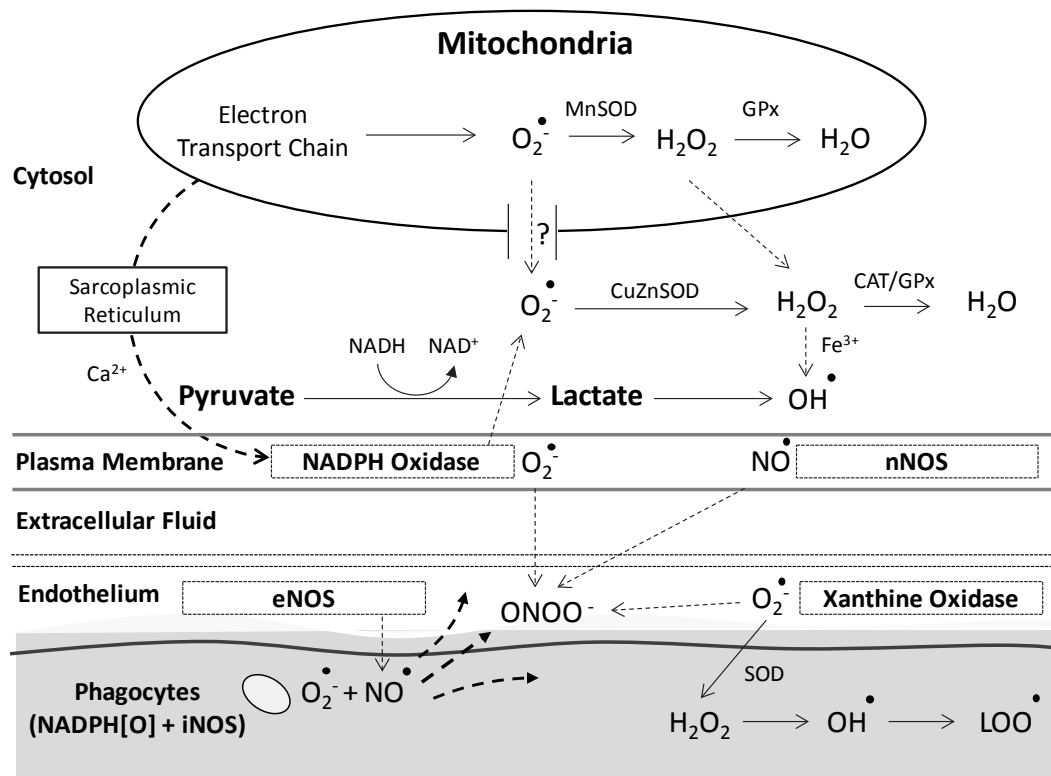


Figure 1.5: Sources of RONS in skeletal muscle. Diagram adapted from Jackson (2000).

The figure indicates the various enzymatic sources of RONS within and around skeletal muscle during exercise. Previously undefined terms: GPx: glutathione peroxidase, Fe^{3+} : ferric ion, Ca^{2+} : calcium ion, CuZnSOD: copper-zinc superoxide dismutase, MnSOD: manganese superoxide dismutase.

1.7.4. Measuring exercise-induced oxidative stress in plasma

There is very well documented evidence that plasma markers of oxidative stress are increased in response to exercise in humans (Turner et al., 2011b; Bloomer et al., 2007;

Goldfarb et al., 2002). Importantly, some studies have reported associations between the magnitude of change in oxidative stress in muscle cells and plasma (You et al., 2005; Nikolaidis et al., 2008). The systemic circulation plays an integral role in maintaining redox balance between tissues (Vollaard et al., 2005). In addition, due to the ease sample collection and isolation, plasma measures of oxidative stress are a less invasive alternative to collecting muscle biopsy samples and isolating cells. Typical measures of exercise-induced oxidative stress include protein carbonyls (Bloomer et al., 2007; Turner et al., 2011c), LOOH (Turner et al., 2011b) and indicators of TAC (Turner et al., 2011c; Berzosa et al., 2011). Whilst alterations in plasma oxidative stress do not reflect oxidative events within muscle that directly stimulate adaptation, monitoring exercise-induced changes to plasma markers of oxidative stress indicate global perturbations that reflect a wide variety of tissues and peripheral cells. Monitoring these changes therefore reflects whole body oxidative modifications.

1.7.5. Measuring exercise-induced PBMC oxidative stress

Exercise-induced oxidative stress is routinely measured in peripheral blood mononuclear cells (PBMCs). PBMCs are composed of lymphocytes ($\approx 90\%$) and monocytes ($\approx 10\%$) and are a 'site' for oxidative modification (Tauler et al., 2006; Sureda et al., 2005) and are a source of ROS during exercise (Sureda et al., 2009). Changes in PBMC adducts represent a more specific indicator of oxidative stress in response to exercise compared to plasma or whole blood, incorporating changes that reflect cellular redox balance and oxidative modifications that impact upon immune function. At rest, PBMCs weakly adhere to the blood vessel wall via CAMS (Albelda et al., 1994). In response to exercise, increases in systemic adrenaline concentrations and exercise-induced shear stress can mobilise PBMCs off the blood vessel wall by activating β_2 -adrenergic receptors (Dimitrov et al., 2009) that

weaken CAMS binding and mechanical dislodgement of PBMCs (Shephard, 2003) respectively. In addition, PBMCs may infiltrate the contracting skeletal muscle via immune regulation of leukocytes to the stressed muscle tissue. Collectively, lymphocytosis is observed during and immediately following exercise as PBMC frequency rapidly increases in peripheral blood by both mobilisation and recruitment based mechanisms (Turner et al., 2011a). An array of studies have reported increases in markers of protein carbonylation (Turner et al., 2011b), lipid oxidation (Sureda et al., 2005) and antioxidant enzyme systems (Sureda et al., 2005; Tauler et al., 2006) following exercise in PBMCs.

1.7.6. Acute increases in inflammation during and following exercise

Exercise is accompanied by an acute rise in both skeletal muscle (Akerstrom et al., 2005; Steensberg et al., 2000) and systemic inflammation (Febbraio et al., 2004; Ostrowski et al., 2001). An increase in inflammatory markers is known to be an immunological response to muscle 'stress' as well as a signalling response for metabolic and transcriptional (i.e. NF- κ B) regulation (Pierce et al., 2009). Inflammatory cytokines are released from multiple cell types during exercise, notably leukocytes, adipocytes and fibroblasts (Akira et al., 1993). In addition, there is accumulating evidence that skeletal muscle can produce muscle-specific cytokine mediators known as 'myokines' that can regulate substrate metabolism and anti-inflammatory transcription (Petersen and Pedersen, 2005). Growing attention is focusing on the interleukin family of cytokines, specifically IL-6, which is thought to act as an 'energy sensing' signal to increase hepatic glucose output (Febbraio et al., 2004, 2003) and adipose tissue lipolysis (van Hall, 2003) during exercise. In addition, IL-6 is known to control the release and transcription of other acute phase proteins such as CRP and anti-inflammatory cytokines, IL-10 and IL-1 receptor antagonist (ra) (Tilg et al., 1994; Steensberg et al., 2003) respectively. IL-10 can suppress pro-inflammatory cytokine production and activation of pro-

inflammatory cells, creating an anti-inflammatory environment (Stevens et al., 2005). Over a period of exercise training, repeated transient increases of cytokines such as IL-6 and IL-10 may signal to lower basal inflammation (Dekker et al., 2007; Adamopoulos et al., 2002). Mechanisms appear to be primarily: reductions in visceral fat mass (a prominent pro-inflammatory cytokines source) (Mujumdar et al., 2011), increased anti-inflammatory cytokine production (Gleeson et al., 2006), phenotypic cell changes and decreased TLR receptor expression (Stewart et al., 2005; Oliveira and Gleeson, 2010). In this manner, inflammatory cytokines share a similar acute/chronic relationship to RONS. Given that these signalling cytokines are released from cells in the circulation (Akira et al., 1993), as well as skeletal muscle (Steensberg et al., 2000), studies routinely assess cytokine concentrations in plasma.

1.8 Hormesis: Understanding the adaptive and pathological roles of oxidative stress and inflammation

1.8.1. Overview

Hormesis is a theory proposing that biological systems respond to radiation, chemicals and toxins with a bell-shaped curve relationship (Rattan, 2001). The theory suggests that there is an ‘optimal’ dose of these stimuli before the beneficial effects on cellular adaptation decline.

1.8.2. Acute exercise-induced oxidative stress and human disease

Radak and colleagues (Radak et al., 2005) extended the concept of hormesis to suggest that the beneficial effects of exercise are in part, related to exercise-induced RONS

production (figure 1.6). The theory is termed the ‘exercise paradox’ and relates to the unknown balance or threshold between RONS that have an adaptive or pathological outcome.

This theory might suggest that in populations with high underlying levels of oxidative stress, that exercise of a prolonged or high intensity might produce levels of RONS that exceed the hormesis threshold and damage cellular biomolecules (Packer, 1997; Radak et al., 1999). Indeed, there is some evidence suggesting that exercise-induced RONS release is related to exercise intensity (Wang and Huang, 2005; Goto et al., 2003) and duration (Bloomer et al., 2007).

Previous studies in patient populations have indicated not only higher baseline differences compared to control but also an exaggerated oxidative stress response (i.e. percentage increase) to an exhaustive bout of exercise (Nishiyama et al., 1998; Avogaro et al., 1986). Whilst the significance of this exaggerated response is not completely clear, these studies demonstrate that there may be an optimal balance between the production of RONS in response to exercise that initiates adaptation and the amount that elicits damage. Interestingly, some previous studies have also highlighted an exaggerated inflammatory response to exercise in patients populations, compared to age-matched controls (Ploeger et al., 2009; Castellano et al., 2008). Exaggerated oxidative stress and inflammatory responses to exercise may be explained by disease associated deteriorations in cellular structure and function, combined with a reduced capacity for the muscle to repair and regenerate (McArdle et al., 2002). Furthermore, the heightened basal levels of oxidative stress and inflammation in patients groups might impair their ability to protect against the stress of the exercise compared to healthy subjects.

Exercise is an extremely beneficial behavioural intervention to improve and optimise health. Evidence suggests that maintaining low basal levels of oxidative stress and inflammation can help extend lifespan and prevent disease. The model of hormesis suggests

that transient exercise-induced increases in RONS are essential in facilitating adaptations that help maintain lower basal levels of oxidative stress over time. However, the dose (i.e. intensity, frequency, duration and modality) of exercise that can achieve optimal adaptations remains unclear. Importantly, this may differ between populations. In diseases associated with inflammation, a fine line may exist between the production of RONS and inflammatory cytokines that can achieve optimal adaptation, and the amount that may cause damage. Monitoring peripheral blood oxidative stress and inflammation in response to various types of exercise, in populations of differing health status' and over periods of training can give us insight into the adaptive and pathological actions of oxidative stress and inflammation in health and disease.

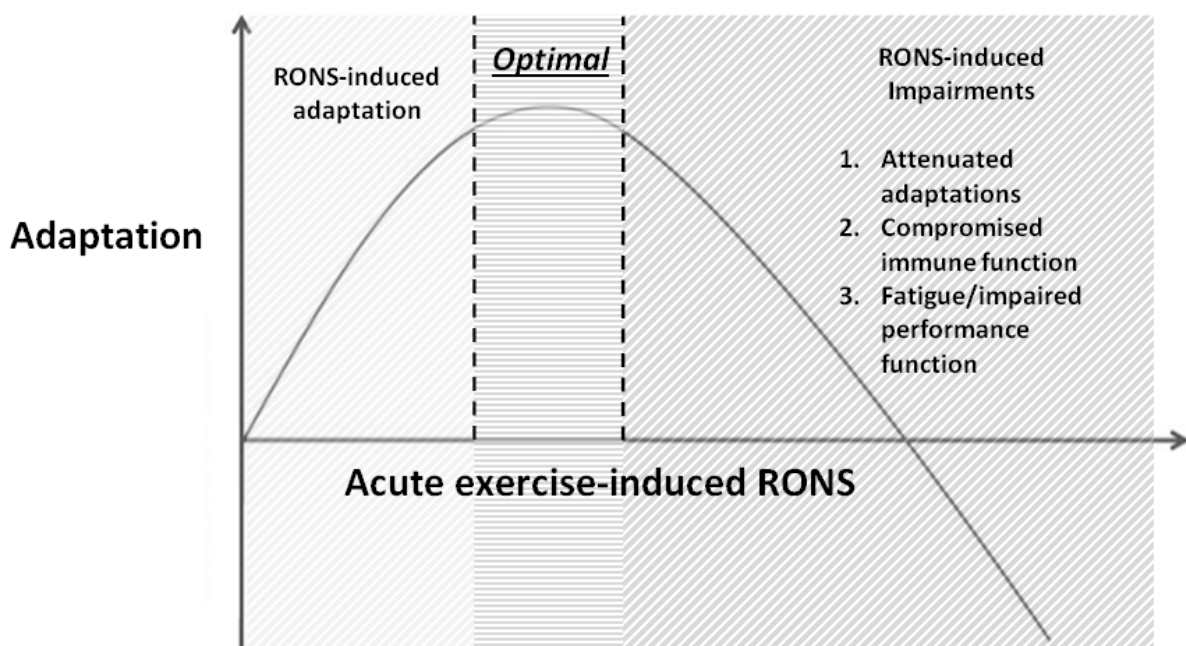


Figure 1.6: Model of Exercise-induced RONS and Hormesis. The theoretical model suggests that there is a threshold whereby certain situations (health status, exercise conditions) may compromise exercise adaptation stimulated by RONS, leading to impaired adaptation and health outcomes.

1.9. Aims of thesis

The aims of this thesis were to explore and characterise acute and chronic changes in markers of oxidative stress and inflammation in response to various stimuli, with a primary focus on exercise. The main objectives were to assess the impact of exercise modality, intensity and health status on these markers. Finally, a consistent aim was to identify/investigate markers that might provide insight into the links between oxidative stress and inflammation. Characterising the perturbations of oxidative stress and inflammation in response to ‘stress’ are essential in aiding understanding of the roles of RONS and inflammatory cytokines in health and disease. Furthermore, this information may facilitate the development of practical suggestions on the use of exercise as a tool to maintain or improve health in a variety of populations. The overarching hypothesis of this thesis was that exercise would induce intensity-dependent changes in markers of oxidative stress and inflammation. It was also hypothesised that exercise in rheumatoid arthritis patients would cause a transient increase in oxidative stress that would aid reductions in baseline oxidative stress over a period of exercise training.

The study presented in *Chapter two* aimed to characterise oxidative stress and inflammation in response to acute mental stress, under conditions of high or basal inflammation. Vaccination and exercise-induced muscle damage were used as experimental interventions to induce inflammation in this study. *Chapter three* aimed to investigate the effects of acute and chronic exercise on markers of oxidative stress and inflammation in patients with rheumatoid arthritis. *Chapter four* looked to characterise acute physiological responses to low volume high intensity interval training (LV-HIIT); a recently designed novel training modality for sedentary and clinical populations. Plasma markers of oxidative stress and inflammation were assessed in response to an acute bout of LV-HIIT and compared with more conventional steady state exercise bouts in healthy participants. Finally, *chapter five*

aimed to establish the response of cellular redox proteins (TRX and PRDX) to LV-HIIT and steady state exercise bouts in healthy participants.

1.10. Reference list for chapter one

- Adamopoulos, S., Parissis, J., Karatzas, D., et al. (2002) Physical training modulates proinflammatory cytokines and the soluble Fas/soluble Fas ligand system in patients with chronic heart failure. **Journal of the American College of Cardiology**, 39 (4): 653–63
- Akerstrom, T., Steensberg, A., Keller, P., et al. (2005) Exercise induces interleukin-8 expression in human skeletal muscle. **J Physiol**, 563 (Pt 2): 507–516
- Akira, S., Taga, T. and Kishimoto, T. (1993) Interleukin-6 in biology and medicine. **Advances in Immunology**, 54: 1–78
- Albelda, S.M., Smith, C.W. and Ward, P.A. (1994) Adhesion molecules and inflammatory injury. **Faseb Journal**, 8: 504–512
- Andrade, F.H., Reid, M.B., Allen, D.G., et al. (1998) Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. **The Journal of physiology**, 509 (2): 565–75
- Aoi, W., Naito, Y., Takanami, Y., et al. (2004) Oxidative stress and delayed-onset muscle damage after exercise. **Free Radical Biology & Medicine**, 37 (4): 480–487
- Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. **Annual review of plant biology**, 55: 373–99
- Avogaro, P., Bon, G.B., Cazzolato, G., et al. (1986) Plasma malondialdehyde in coronary patients and in “normal” people at rest and after exercise. **Texas Heart Institute journal / from the Texas Heart Institute of St. Luke’s Episcopal Hospital, Texas Children’s Hospital**, 13 (3): 291–6
- Babior, B.M. (2000) Phagocytes and oxidative stress. **American Journal of Medicine**, 109 (1): 33–44
- Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. **Journal of Clinical Investigation**, 52 (3): 741–744
- Bailey, S.J., Vanhatalo, A., Winyard, P.G., et al. (2012) The nitrate-nitrite oxide pathway: Its role in human exercise physiology. **European Journal of Sport Science**, 12 (4): 309–320
- Bains, J.S. and Shaw, C. A (1997) Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. **Brain research. Brain research reviews**, 25 (3): 335–58

Balon, T.W. and Nadler, J.L. (1994) Nitric oxide release is present from incubated skeletal muscle preparations. **Journal of Applied Physiology**, 77 (6): 2519–2521

Beckman, J.S. (1996) Oxidative damage and tyrosine nitration from peroxynitrite. **Chemical Research in Toxicology**, 9 (5): 836–844

Beckman, J.S., Beckman, T.W., Chen, J., et al. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. **Proc Natl Acad Sci U S A**, 87 (4): 1620–1624

Beckman, J.S. and Koppenol, W.H. (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. **The American journal of physiology**, 271 (5): C1424–37

Benhar, M., Forrester, M.T., Hess, D.T., et al. (2008) Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. **Science (New York, N.Y.)**, 320 (5879): 1050–4

Benhar, M., Thompson, J.W., Moseley, M.A., et al. (2010) Identification of S-nitrosylated targets of thioredoxin using a quantitative proteomic approach. **Biochemistry**, 49 (32): 6963–9

Benjamin, N., O'Driscoll, F., Dougall, et al. (1994). Stomach NO synthesis. **Nature**, 368: 502–503

Bennett, S., Grant, M.M. and Aldred, S. (2009) Oxidative stress in vascular dementia and Alzheimer's disease: a common pathology. **Journal of Alzheimers Disease**, 17 (2): 245–257

Berzosa, C., Cebrian, I., Fuentes-Broto, L., et al. (2011) Acute exercise increases plasma total antioxidant status and antioxidant enzyme activities in untrained men. **J Biomed Biotechnol**, pp. 1–7

Bloomer, R.J., Davis, P.G., Consitt, L.A., et al. (2007) Plasma protein carbonyl response to increasing exercise duration in aerobically trained men and women. **International Journal of Sports Medicine**, 28 (1): 21–25

Bloomer, R.J., Goldfarb, A.H., Wideman, L., et al. (2005) Effects of acute aerobic and anaerobic exercise on blood markers of oxidative stress. **Journal of strength and conditioning research / National Strength & Conditioning Association**, 19 (2): 276–85

Brandes, R.P., Weissmann, N. and Schröder, K. (2010) NADPH oxidases in cardiovascular disease. **Free radical biology & medicine**, 49 (5): 687–706

Breusing, N., Arndt, J., Voss, P., et al. (2009) Inverse correlation of protein oxidation and proteasome activity in liver and lung. **Mechanisms of ageing and development**, 130 (11–12): 748–53

Breusing, N., Grune, T., Andrisic, L., et al. (2010) An inter-laboratory validation of methods of lipid peroxidation measurement in UVA-treated human plasma samples. **Free Radic Res**, 44 (10): 1203–1215

Bruunsgaard, H. (2005) Physical activity and modulation of systemic low-level inflammation. **J Leukoc Biol**, 78 (4): 819–835

Buettner, G.R. (1993) The pecking order of free radicals and antioxidants lipid peroxidation,.pdf. **Archives of Biochemistry and Biophysics**, 300 (2): 535–543

Burke-Gaffney, A., Callister, M.E. and Nakamura, H. (2005) Thioredoxin: friend or foe in human disease? **Trends Pharmacol Sci**, 26 (8): 398–404

Buss, H., Chan, T.P., Sluis, K.B., et al. (1997) Protein carbonyl measurement by a sensitive ELISA method. **Free Radical Biology and Medicine**, 23 (3): 361–366

Butterfield, D.A., Perluigi, M. and Sultana, R. (2006) Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics. **Eur J Pharmacol**, 545 (1): 39–50

Cai, H. and Harrison, D.G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. **Circ Res**, 87 (10): 840–844

Cao, G. and Prior, R.L. (1998) Comparison of different analytical methods for assessing total antioxidant capacity of human serum. **Clinical chemistry**, 44 (6 Pt 1): 1309–15

Carty, J.L., Bevan, R., Waller, H., et al. (2000) The effects of vitamin C supplementation on protein oxidation in healthy volunteers. **Biochem Biophys Res Commun**, 273 (2): 729–735

Castellano, V., Patel, D.I. and White, L.J. (2008) **Cytokine responses to acute and chronic exercise in multiple sclerosis.**, 27513: 1697–1702

Ceriello, A. and Motz, E. (2004) Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. **Arterioscler Thromb Vasc Biol**, 24 (5): 816–823

Choi, J., Malakowsky, C.A., Talent, J.M., et al. (2002) Identification of oxidized plasma proteins in Alzheimer's disease. **Biochemical and Biophysical Research Communications**, 293: 1566–1570

Clarkson, P., Montgomery, H.E., Mullen, M.J., et al. (1999) Exercise training enhances endothelial function in young men. **Journal of the American College of Cardiology**, 33 (5): 1379–85

Colbert, L.H., Visser, M., Simonsick, E.M., et al. (2004) Physical activity, exercise, and inflammatory markers in older adults: findings from the Health, Aging and Body Composition Study. **J Am Geriatr Soc**, 52 (7): 1098–1104

- Cox, C.J., Habermann, T.M., Payne, B.A., et al. (1985) Evaluation of the Coulter Counter model S-Plus IV. **American Journal of Clinical Pathology**, 84 (3): 297–306
- Dalle-Donne, I., Rossi, R., Giustarini, D., et al. (2003) Protein carbonyl groups as biomarkers of oxidative stress. **Clin Chim Acta**, 329 (1-2): 23–38
- Davies, K.J.A., Quintanilha, A.T., Brooks, G.A., et al. (1982) Free radicals and tissue damage produced by exercise. **Biochemical and Biophysical Research Communications**, 107 (4): 1198–1205
- Dekker, M.J., Lee, S., Hudson, R., et al. (2007) An exercise intervention without weight loss decreases circulating interleukin-6 in lean and obese men with and without type 2 diabetes mellitus. **Metabolism: clinical and experimental**, 56 (3): 332–8
- Dekkers, J.C., van Doornen, L.J. and Kemper, H.C. (1996) The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. **Sports medicine (Auckland, N.Z.)**, 21 (3): 213–38
- Devaraj, S., Dasu, M.R., Singh, U., et al. (2009) C-reactive protein stimulates superoxide anion release and tissue factor activity in vivo. **Atherosclerosis**, 203 (1): 67–74
- Dhalla, N.S., Temsah, R.M. and Netticadan, T. (2000) Role of oxidative stress in cardiovascular diseases. **Journal of Hypertension**, 18 (6): 655–673
- Dimitrov, S., Benedict, C., Heutling, D., et al. (2009) Cortisol and epinephrine control opposing circadian rhythms in T cell subsets. **Blood**, 113 (21): 5134–43
- Donato, A.J., Pierce, G.L., Lesniewski, L.A., et al. (2009) Role of NFκB in age-related vascular endothelial dysfunction in humans. **Aging (Albany NY)**, 1 (8): 678–680
- Donato, A.J., Uberoi, A., Bailey, D.M., et al. (2010) Exercise-induced brachial artery vasodilation: effects of antioxidants and exercise training in elderly men. **Am J Physiol Heart Circ Physiol**, 298 (2): H671–8
- Engelhart, M.J., Geerlings, M.I., Meijer, J., et al. (2004) Inflammatory proteins in plasma and the risk of dementia: the rotterdam study. **Arch Neurol**, 61 (5): 668–672
- Fatouros, I.G., Jamurtas, A.Z., Villiotou, V., et al. (2004) Oxidative Stress Responses in Older Men during Endurance Training and Detraining. **Medicine & Science in Sports & Exercise**, (24): 2065–2072
- Febbraio, M.A., Hiscock, N., Sacchetti, M., et al. (2004) Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction. **Diabetes**, 53 (7): 1643–8

- Febbraio, M.A., Ott, P., Nielsen, H.B., et al. (2003) Hepatosplanchnic clearance of interleukin-6 in humans during exercise. **American journal of physiology Endocrinology and metabolism**, 285 (2): E397–402
- Febbraio, M.A., Rose-John, S. and Pedersen, B.K. (2010) Is interleukin-6 receptor blockade the Holy Grail for inflammatory diseases? **Clinical pharmacology and therapeutics**, 87 (4): 396–8
- Fischer, C.P., Hiscock, N.J., Penkowa, M., et al. (2004) Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle. **The Journal of physiology**, 558 (Pt 2): 633–45
- Fisher-Wellman, K. and Bloomer, R.J. (2009) Acute exercise and oxidative stress: a 30 year history. **Dynamic medicine**, 8: 1–25
- Fu, Y., Luo, N. and Lopes-Virella, M.F. (2002) Upregulation of interleukin-8 expression by prostaglandin D2 metabolite 15-deoxy-delta12, 14 prostaglandin J2 (15d-PGJ2) in human THP-1 macrophages. **Atherosclerosis**, 160 (1): 11–20
- Fuhrman, B., Lavy, A. and Aviram, M. (1995) Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. **The American journal of clinical nutrition**, 61 (3): 549–54
- Gabay, C. and Kushner, I. (1999) Acute Phase Proteins and other systemic responses to inflammation. **The New England Journal of Medicine**, 11: 448–454
- Gleeson, M., Bishop, N.C., Stensel, D.J., et al. (2011) **The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease.**, 11: 607–615
- Gleeson, M., McFarlin, B. and Michael Flynn (2006) Exercise and Toll-like receptors. **Exerc. Immunol. Rev**, 12: 34–53
- Gloire, G., Legrand-Poels, S. and Piette, J. (2006) NF-kappaB activation by reactive oxygen species: fifteen years later. **Biochemical pharmacology**, 72 (11): 1493–505
- Go, Y.M., Ziegler, T.R., Johnson, J.M., et al. (2007) Selective protection of nuclear thioredoxin-1 and glutathione redox systems against oxidation during glucose and glutamine deficiency in human colonic epithelial cells. **Free Radic Biol Med**, 42 (3): 363–370
- Goldfarb, A.H., Bloomer, R.J. and McKenzie, M.J. (2005) Combined Antioxidant Treatment Effects on Blood Oxidative Stress after Eccentric Exercise. **Medicine & Science in Sports & Exercise**, 37 (2): 234–239

Goldfarb, A.H., McIntosh, M.K., Boyer, B.T., et al. (1994) Vitamin E effects on indexes of lipid peroxidation in muscle from DHEA-treated and exercised rats. **Journal of applied physiology (Bethesda, Md. : 1985)**, 76 (4): 1630–5

Goldfarb, A.H., You, T., Bloomer, R.J., et al. (2002) Blood oxidative stress markers in response to aerobic exercise: effect of gender. **Med Sci Sports Exerc**, 34: S429

Gomez-Cabrera, M.C., Domenech, E., Romagnoli, M., et al. (2008) Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. **The American journal of clinical nutrition**, 87 (1): 142–9

Goto, C., Higashi, Y., Kimura, M., et al. (2003) Effect of different intensities of exercise on endothelium-dependent vasodilation in humans: role of endothelium-dependent nitric oxide and oxidative stress. **Circulation**, 108 (5): 530–5

Goto, S., Naito, H., Kaneko, T., et al. (2007) Hormetic effects of regular exercise in aging: correlation with oxidative stress. **Appl Physiol Nutr Metab**, 32 (5): 948–953

Griffiths, H.R., Aldred, S., Dale, C., et al. (2006) Homocysteine from endothelial cells promotes LDL nitration and scavenger receptor uptake. **Free Radical Biology and Medicine**, 40 (3): 488–500

Guzik, T.J., Korbout, R. and Adamek-Guzik, T. (2003) Nitric oxide and superoxide in inflammation and immune regulation. **J Physiol Pharmacol**, 54 (4): 469–487

Van Hall, G. (2003) Interleukin-6 Stimulates Lipolysis and Fat Oxidation in Humans. **Journal of Clinical Endocrinology & Metabolism**, 88 (7): 3005–3010

Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry. **J Gerontol**, 11 (3): 298–300

Harman, D. (2006) Free radical theory of aging: an update: increasing the functional life span. **Ann N Y Acad Sci**, 1067: 10–21

Hart, M.L., Walsh, M.C. and Stahl, G.L. (2004) Initiation of complement activation following oxidative stress. In vitro and in vivo observations. **Mol Immunol**, 41 (2-3): 165–171

Hart, N., Sarga, L., Csende, Z., et al. (2013) Resveratrol Attenuates Exercise-Induced Adaptive Responses in Rats Selectively Bred for Low Running Performance. **Dose-Response**, 1 (-1): 1–15

Herce-Pagliai, C., Kotecha, S. and Shuker, D.E. (1998) Analytical methods for 3-nitrotyrosine as a marker of exposure to reactive nitrogen species: a review. **Nitric Oxide**, 2 (5): 324–336

- Ishii, T., Warabi, E. and Yanagawa, T. (2012) **Novel roles of peroxiredoxins in inflammation , cancer and innate immunity.**, 50 (2): 91–105
- Jackson, M.J. (2005) Reactive oxygen species and redox-regulation of skeletal muscle adaptations to exercise. **Philos Trans R Soc Lond B Biol Sci**, 360 (1464): 2285–2291
- Jackson, M.J. (2008) Redox regulation of skeletal muscle. **IUBMB Life**, 60 (8): 497–501
- Jackson, M.J., Jones, D.A. and Edwards, R.H. (1983) Vitamin E and skeletal muscle. **Ciba Found Symp**, 101: 224–239
- Jackson, M.J., Khassaf, M., Vasilaki, a, et al. (2004) Vitamin E and the oxidative stress of exercise. **Annals of the New York Academy of Sciences**, 1031: 158–68
- Jacob, C. and Ba, L.A. (2011) Open Season for Hunting and Trapping Post-translational Cysteine Modifications in Proteins and Enzymes. **Chembiochem**, 12 (6): 841–844
- Ji, L.L. (2001) Exercise at old age: does it increase or alleviate oxidative stress? **Ann N Y Acad Sci**, 928: 236–247
- Kalsheker, N. a (1994) Molecular pathology of alpha 1-antitrypsin deficiency and its significance to clinical medicine. **QJM : monthly journal of the Association of Physicians**, 87 (11): 653–8
- Kaplanski, G., Marin, V., Montero-Julian, F., et al. (2003) IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. **Trends Immunol**, 24 (1): 25–29
- Khassaf, M., McArdle, A., Esanu, C., et al. (2003) Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. **Journal of Physiology**, 549 (2): 645–652
- Kim, J.A., Montagnani, M., Koh, K.K., et al. (2006) Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. **Circulation**, 113 (15): 1888–1904
- Kim, Y.M., Bombeck, C.A. and Billiar, T.R. (1999) Nitric oxide as a bifunctional regulator of apoptosis. **Circ Res**, 84 (3): 253–256
- Kondo, M., Oya-Ito, T., Kumagai, T., et al. (2001) Cyclopentenone prostaglandins as potential inducers of intracellular oxidative stress. **Journal of Biological Chemistry**, 276 (15): 12076–12083
- Kong, S., Yim, M.B., Stadtman, E.R., et al. (1996) Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: Lymphocyte-specific tyrosine kinase fails to

phosphorylate nitrated cdc2(6-20)NH₂ peptide. **Proc Natl Acad Sci U S A**, 93 (April): 3377–3382

Lane, T., Flam, B., Lockey, R., et al. (2013) TXNIP shuttling: missing link between oxidative stress and inflammasome activation. **Frontiers in physiology**, 4 (March): 50

Lee, D.M. and Weinblatt, M.E. (2001) Rheumatoid arthritis. **Lancet**, 358 (9285): 903–911

Levine, R.L., Williams, J.A., Stadtman, E.R., et al. (1994) Carbonyl assays for determination of oxidatively modified proteins. **Methods Enzymol**, 233: 346–357

Lewis, T.V., Dart, A.M., Chin-Dusting, J.P., et al. (1999) Exercise training increases basal nitric oxide production from the forearm in hypercholesterolemic patients. **Arterioscler Thromb Vasc Biol**, 19 (11): 2782–2787

Li, J.M. and Shah, A.M. (2004) Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. **American Journal of Physiology-Regulatory Integrative and Comparative Physiology**, 287 (5): R1014–R1030

Libby, P. (2008) Role of inflammation in atherosclerosis associated with rheumatoid arthritis. **Am J Med**, 121 (10 Suppl 1): S21–31

Lundberg, J.O. and Weitzberg, E. (2009) NO generation from inorganic nitrate and nitrite: Role in physiology, nutrition and therapeutics. **Arch Pharm Res**, 32 (8): 1119–1126

Mapp, P.I., Grootveld, M.C. and Blake, D.R. (1995) Hypoxia, oxidative stress and rheumatoid arthritis. **Br Med Bull**, 51 (2): 419–436

Martin, G.M., Austad, S.N. and Johnson, T.E. (1996) Genetic analysis of ageing: role of oxidative damage and environmental stresses. **Nature Genetics**, 13: 25–34

May, J.M., Mendiratta, S., Hill, K.E., et al. (1997) Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. **Journal of Biological Chemistry**, 272 (36): 22607–22610

Mayrovitz, H.N., Wiedman, M.P. and Tuma, R.F. (1977) Factors influencing leukocyte adherence in microvessels. **Thromb Haemost**, 38 (4): 823–830

McArdle, A., Pattwell, D., Vasilaki, A., et al. (2001) Contractile activity-induced oxidative stress: cellular origin and adaptive responses. **Am J Physiol Cell Physiol**, 280 (3): C621–7

McArdle, A., Vasilaki, A. and Jackson, M. (2002) Exercise and skeletal muscle ageing: cellular and molecular mechanisms. **Ageing Res Rev**, 1 (1): 79–93

- McArdle, F., Pattwell, D.M., Vasilaki, A., et al. (2005) Intracellular generation of reactive oxygen species by contracting skeletal muscle cells. **Free radical biology & medicine**, 39 (5): 651–7
- Medzhitov, R. (2008) Origin and physiological roles of inflammation. **Nature**, 454 (7203): 428–435
- Metsios, G.S., Stavropoulos-Kalinoglou, A., Sandoo, A., et al. (2010) Vascular function and inflammation in rheumatoid arthritis: the role of physical activity. **Open Cardiovasc Med J**, 4: 89–96
- Metsios, G.S., Stavropoulos-Kalinoglou, A., Veldhuijzen van Zanten, J.J.C.S., et al. (2008) Rheumatoid arthritis, cardiovascular disease and physical exercise: a systematic review. **Rheumatology (Oxford)**, 47 (3): 239–248
- Michailidis, Y., Jamurtas, A.Z., Nikolaidis, M.G., et al. (2007) Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress. **Medicine and science in sports and exercise**, 39 (7): 1107–13
- Moraga, F. (2000) Activation of Primary Human Monocytes by the Oxidized Form of alpha 1-Antitrypsin. **Journal of Biological Chemistry**, 275 (11): 7693–7700
- Moshage, H., Kok, B., Huizenga, J.R., et al. (1995) Nitrite and nitrate determinations in plasma: a critical evaluation. **Clin Chem**, 41 (6 Pt 1): 892–896
- Mujumdar, P.P., Duerksen-Hughes, P.J., Firek, A.F., et al. (2011) Long-term, progressive, aerobic training increases adiponectin in middle-aged, overweight, untrained males and females. **Scandinavian Journal of Clinical & Laboratory Investigation**, 71 (2): 101–107
- Naik, E. and Dixit, V.M. (2011) Mitochondrial reactive oxygen species drive pro-inflammatory cytokine production. **The Journal of experimental medicine**, 208 (3): 417–20
- Niki, E. (2009) Lipid peroxidation: physiological levels and dual biological effects. **Free Radic Biol Med**, 47 (5): 469–484
- Nikolaidis, M.G., Jamurtas, A.Z., Paschalis, V., et al. (2008) The effect of muscle-damaging exercise on blood and skeletal muscle oxidative stress: magnitude and time-course considerations. **Sports Med**, 38 (7): 579–606
- Nikolaidis, M.G., Kyparos, A. and Vrabas, I.S. (2011) F-isoprostane formation, measurement and interpretation: the role of exercise. **Prog Lipid Res**, 50 (1): 89–103
- Nishiyama, Y., Ikeda, H., Haramaki, N., et al. (1998) Oxidative stress is related to exercise intolerance in patients with heart failure. **American Heart Journal**, 135: 115–120

- Nordberg, J. and Arner, E.S. (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. **Free Radic Biol Med**, 31 (11): 1287–1312
- O'Neill, C.A., Stebbins, C.L., Bonigut, S., et al. (1996) Production of hydroxyl radicals in contracting skeletal muscle of cats. **J Appl Physiol**, 81 (3): 1197–1206
- Oliveira, M. and Gleeson, M. (2010) The influence of prolonged cycling on monocyte Toll-like receptor 2 and 4 expression in healthy men. **European journal of applied physiology**, 109 (2): 251–7
- Ostrowski, K., Rohde, T., Asp, S., et al. (2001) Chemokines are elevated in plasma after strenuous exercise in humans. **European journal of applied physiology**, 84 (3): 244–245
- Packer, L. (1997) Oxidants, antioxidant nutrients and the athlete. **J Sports Sci**, 15 (3): 353–363
- Parratt, J.R. (1997) Nitric oxide. A key mediator in sepsis and endotoxaemia? **J Physiol Pharmacol.**, 48 (4): 493–506
- Patwari, P., Higgins, L.J., Chutkow, W.A., et al. (2006) The interaction of thioredoxin with Txnip. Evidence for formation of a mixed disulfide by disulfide exchange. **Journal of Biological Chemistry**, 281 (31): 21884–21891
- Petersen, A.M.W. and Pedersen, B.K. (2005) The anti-inflammatory effect of exercise. **J Appl Physiol**, 98 (4): 1154–1162
- Pierce, G.L., Lesniewski, L.A., Lawson, B.R., et al. (2009) Nuclear factor- κ B activation contributes to vascular endothelial dysfunction via oxidative stress in overweight/obese middle-aged and older humans. **Circulation**, 119 (9): 1284–1292
- Ploeger, H.E., Takken, T., de Greef, M.H.G., et al. (2009) The effects of acute and chronic exercise on inflammatory markers in children and adults with a chronic inflammatory disease: a systematic review. **Exercise immunology review**, 15: 6–41
- Powers, S.K. and Jackson, M.J. (2008) Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. **Physiol Rev**, 88 (4): 1243–1276
- Pryor, W.A. and Squadrito, G.L. (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. **American Journal of Physiology**, 268 (5 Pt 1): L699–722
- Quehenberger, O., Armando, A.M., Brown, A.H., et al. (2010) Lipidomics reveals a remarkable diversity of lipids in human plasma. **Journal of lipid research**, 51 (11): 3299–305

- Radak, Z., Chung, H.Y. and Goto, S. (2005) Exercise and hormesis: oxidative stress-related adaptation for successful aging. **Biogerontology**, 6 (1): 71–5
- Radak, Z., Kaneko, T., Tahara, S., et al. (1999) The effect of exercise training on oxidative damage of lipids, proteins, and DNA in rat skeletal muscle: evidence for beneficial outcomes. **Free Radic Biol Med**, 27 (1-2): 69–74
- Rattan, S.I.S. (2001) Applying hormesis in aging research and therapy. **Human & Experimental Toxicology**, 20 (6): 281–285
- Reznick, A.Z. and Packer, L. (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. **Methods in Enzymology**, 223: 357–363
- Rhee, S.G., Woo, H.A., Kil, I.S., et al. (2012) Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides. **The Journal of biological chemistry**, 287 (7): 4403–10
- Riddell, J.R., Wang, X.Y., Minderman, H., et al. (2010) Peroxiredoxin 1 stimulates secretion of proinflammatory cytokines by binding to TLR4. **Journal of immunology (Baltimore, Md. : 1950)**, 184 (2): 1022–30
- Ristow, M., Birringer, M., Kiehntopf, M., et al. (2009) Antioxidants prevent health-promoting effects. **PNAS**, 106 (21): 8865–8670
- Salo, D.C., Donovan, C.M. and Davies, K.J.A. (1991) HSP70 and other possible heat shock or oxidative stress proteins are induced in skeletal muscle, heart, and liver during exercise. **Free Radical Biology and Medicine**, 11 (3): 239–246
- Sarban, S., Kocyigit, A., Yazar, M., et al. (2005) Plasma total antioxidant capacity, lipid peroxidation, and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. **Clinical biochemistry**, 38 (11): 981–6
- Schenk, H., Klein, M., Erdbrügger, W., et al. (1994) Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. **Proc Natl Acad Sci U S A**, 91 (5): 1672–1676
- Schenk, H., Vogt, M., Droge, W., et al. (1996) Thioredoxin as a potent costimulus of cytokine expression. **J Immunol**, 156 (2): 765–771
- Schulze, P.C., Liu, H., Choe, E., et al. (2006) Nitric oxide-dependent suppression of thioredoxin-interacting protein expression enhances thioredoxin activity. **Arterioscler Thromb Vasc Biol**, 26 (12): 2666–2672
- Seven, A., Guzel, S., Aslan, M., et al. (2008) Lipid, protein, DNA oxidation and antioxidant status in rheumatoid arthritis. **Clinical Biochemistry**, 41 (7-8): 538–543

- Shephard, R.J. (2003) Adhesion molecules, catecholamines and leucocyte redistribution during and following exercise. **Sports medicine (Auckland, N.Z.)**, 33 (4): 261–84
- Shephard, R.J. and Balady, G.J. (1999) Exercise as cardiovascular therapy. **Circulation**, 99 (7): 963–972
- Singh, T. and Newman, A.B. (2010) Inflammatory markers in population studies of aging. **Ageing Res Rev**
- Smith, W.L. (1989) Prostaglandin growth and differentiation. **Biochem. J.**, 254: 315–324
- Souza, J.M., Peluffo, G. and Radi, R. (2008) Protein tyrosine nitration--functional alteration or just a biomarker? **Free radical biology & medicine**, 45 (4): 357–66
- Stadtman, E.R. and Berlett, B.S. (1997) Reactive oxygen-mediated protein oxidation in aging and disease. **Chem Res Toxicol**, 10 (5): 485–494
- Stain, C., Stockinger, H., Scharf, M., et al. (2013) Human blood basophils display a unique phenotype including activation linked membrane structures. **Blood**, 7 (6): 1872–1879
- Steensberg, A., Fischer, C.P., Keller, C., et al. (2003) IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. **American journal of physiology. Endocrinology and metabolism**, 285 (2): E433–7
- Steensberg, A., van Hall, G., Osada, T., et al. (2000) Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. **The Journal of physiology**, 529 Pt 1: 237–42
- Stevens, R.J., Douglas, K.M., Saratzis, A.N., et al. (2005) Inflammation and atherosclerosis in rheumatoid arthritis. **Expert Rev Mol Med**, 7 (7): 1–24
- Stewart, L.K., Flynn, M.G., Campbell, W.W., et al. (2005) Influence of exercise training and age on CD14+ cell-surface expression of toll-like receptor 2 and 4. **Brain, behavior, and immunity**, 19 (5): 389–97
- Sureda, A., Ferrer, M.D., Tauler, P., et al. (2009) Effects of exercise intensity on lymphocyte H₂O₂ production and antioxidant defences in soccer players. **Med, Br J Sports**, 43: 186–190
- Sureda, A., Tauler, P., Aguiló, A., et al. (2005) Relation between oxidative stress markers and antioxidant endogenous defences during exhaustive exercise. **Free radical research**, 39 (12): 1317–24
- Szabó-Taylor, K.É., Eggleton, P., Turner, C.A.L., et al. (2012) Lymphocytes from rheumatoid arthritis patients have elevated levels of intracellular peroxiredoxin 2, and a greater frequency of cells with exofacial peroxiredoxin 2, compared with healthy human lymphocytes. **The international journal of biochemistry & cell biology**, 44 (8): 1223–31

- Tan, S.X.X., Greetham, D., Raeth, S., et al. (2010) The thioredoxin-thioredoxin reductase system can function in vivo as an alternative system to reduce oxidized glutathione in *Saccharomyces cerevisiae*. **Journal of Biological Chemistry**, 285 (9): 6118–6126
- Tauler, P., Sureda, A., Cases, N., et al. (2006) Increased lymphocyte antioxidant defences in response to exhaustive exercise do not prevent oxidative damage. **The Journal of nutritional biochemistry**, 17 (10): 665–71
- Taysi, S., Polat, F., Gul, M., et al. (2002) Lipid peroxidation, some extracellular antioxidants, and antioxidant enzymes in serum of patients with rheumatoid arthritis. **Rheumatol Int**, 21 (5): 200–204
- Tilg, H., Trehu, E., Atkins, M.B., et al. (1994) Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. **Blood**, 83 (1): 113–8
- Tracy, R.P. (1998) Inflammation in cardiovascular disease: cart, horse, or both? **Circulation**, 97 (20): 2000–2002
- Turner, J.E., Bosch, J.A. and Aldred, S. (2011a) Measurement of exercise-induced oxidative stress in lymphocytes. **Biochemical Society transactions**. 39 (5) pp. 1299–304
- Turner, J.E., Bosch, J.A., Drayson, M.T., et al. (2011b) Assessment of oxidative stress in lymphocytes with exercise. **Journal of applied physiology (Bethesda, Md. : 1985)**, 111 (1): 206–11
- Turner, J.E., Hodges, N.J., Bosch, J.A., et al. (2011c) Prolonged Depletion of Antioxidant Capacity after Ultraendurance Exercise. **Medicine & Science in Sports & Exercise**, 43 (9): 1770–1776
- Ungvari, Z., Kaley, G., de Cabo, R., et al. (2010) Mechanisms of vascular aging: new perspectives. **J Gerontol A Biol Sci Med Sci**, 65 (10): 1028–1041
- Utgaard, J.O., Jahnsen, F.L., Bakka, A., et al. (1998) Rapid secretion of prestored interleukin 8 from Weibel-Palade bodies of microvascular endothelial cells. **J Exp Med**, 188 (9): 1751–1756
- Vassilakopoulos, T., Karatza, M.-H., Katsaounou, P., et al. (2003) Antioxidants attenuate the plasma cytokine response to exercise in humans. **Journal of applied physiology (Bethesda, Md. : 1985)**, 94 (3): 1025–32
- Vollaard, N.B., Shearman, J.P. and Cooper, C.E. (2005) Exercise-induced oxidative stress: myths, realities and physiological relevance. **Sports Med**, 35 (12): 1045–1062

- Wadley, A.J., Chen, Y.W., Lip, G.Y.H., Fisher, J.P., et al. (2013) Effect of low volume high intensity interval and steady state exercise bouts on oxidative stress and inflammation in humans. **Med Sci Sports Exerc**, (under review)
- Wang, J.-S. and Huang, Y.-H. (2005) Effects of exercise intensity on lymphocyte apoptosis induced by oxidative stress in men. **European journal of applied physiology**, 95 (4): 290–7
- Wiedenheft, B., Mosolf, J., Willits, D., et al. (2005) An archaeal antioxidant: characterization of a Dps-like protein from *Sulfolobus solfataricus*. **Proceedings of the National Academy of Sciences of the United States of America**, 102 (30): 10551–6
- Willerson, J.T. and Ridker, P.M. (2004) Inflammation as a cardiovascular risk factor. **Circulation**, 109 (21 Suppl 1): II2–10
- Wood, Z. A, Poole, L.B. and Karplus, P.A. (2003) Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. **Science (New York, N.Y.)**, 300 (5619): 650–3
- World, C., Spindel, O.N. and Berk, B.C. (2011) Thioredoxin-interacting protein mediates TRX1 translocation to the plasma membrane in response to tumor necrosis factor- α : a key mechanism for vascular endothelial growth factor receptor-2 transactivation by reactive oxygen species. **Arterioscler Thromb Vasc Biol**, 31 (8): 1890–1897
- Yamada, Y., Nakamura, H., Adachi, T., et al. (2003) Elevated serum levels of thioredoxin in patients with acute exacerbation of asthma. **Immunol Lett**, 86 (2): 199–205
- You, T., Goldfarb, A.H., Bloomer, R., et al. (2005) Oxidative Stress Response in Normal and Antioxidant Supplemented Rats to a Downhill Run: Changes in Blood and Skeletal Muscle. **Canadian Journal of Applied Physiology**, 30 (6): 677–689
- Yudkin, J.S. (2007) Inflammation, obesity, and the metabolic syndrome. **Hormone Metabolism Research**, 39: 707–9
- Zhou, R., Tardivel, A., Thorens, B., et al. (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. **Nat Immunol**, 11 (2): 136–140

Chapter Two

2. Underlying inflammation has no impact on the oxidative stress response to acute mental stress

2.1. Abstract

Introduction: Mental stress is considered to be a trigger for acute myocardial infarction (MI), with inflammation thought to provide a mechanism. Inflammation is reciprocally linked to oxidative stress, which has also been implicated in MI. The purpose of this study was to assess the effects of experimentally-induced inflammation on the oxidative stress response to mental stress in healthy participants.

Methods: Healthy males undertook one of two inflammatory stimuli: typhoid vaccination (Vaccination paradigm, N=17) or eccentric exercise (Eccentric exercise paradigm, N=17). All participants completed a mental arithmetic stress task twice: six hours after the inflammatory stimulus, and during a control non-inflammation condition. Blood samples were taken before, immediately and thirty minutes after the stress task. Plasma was assessed for interleukin-6 (IL-6), protein carbonyls (PC), lipid hydroperoxides (LOOH), total antioxidant capacity (TAC) and nitric oxide metabolites (NOx).

Results: *Vaccination paradigm:* IL-6, PC and NOx were significantly higher in the vaccination condition, relative to the control condition ($p<.05$). PC, TAC, LOOH and NOx were unchanged in response to mental stress in both the vaccination and control conditions. *Eccentric Exercise paradigm:* IL-6 and TAC were significantly higher in the eccentric exercise condition ($p<.05$), relative to the control condition. PC, TAC and NOx were unchanged in response to mental stress in both the eccentric exercise and control conditions.

Conclusions: Two different inflammatory paradigms were successful in increasing plasma markers of inflammation and oxidative stress prior to a mental stress task. However, experimentally induced transient inflammation had no impact on mental stress-induced changes in plasma LOOH, PC, TAC or NOx in young healthy participants.

2.2. Introduction

Mental stress has been proposed as a potential trigger for myocardial infarction (MI) (Strike and Steptoe, 2005; Bergovec et al., 1992). The physiologic mechanisms linking mental stress to MI have not been fully clarified, however inflammation has been implicated (Wilbert-Lampen et al., 2010; Paine and Veldhuijzen van Zanten, 2012). Laboratory models have been used to investigate the role of inflammation in populations with elevated risk for cardiovascular disease and findings have shown that mental stress-induced ischemia is associated with future MI (Babyak et al., 2011; Krantz et al., 1999). Interestingly, mental stress-induced ischemia is more prevalent in those with higher baseline levels of inflammation (Shah et al., 2006). Inflammation is known to be intrinsically linked to oxidative stress, a state whereby the production of reactive oxygen species (ROS) overwhelms antioxidants (Wadley et al., 2012). Both oxidative stress and inflammation are independently associated with the development of cardiovascular disease (CVD) and MI (Cai and Harrison, 2000; Dhalla et al., 2000; Ross, 1999). Given the adverse effect of oxidative stress on MI risk, the relationship between mental stress and oxidative stress warrants further investigation.

The few studies that have investigated the links between oxidative stress and acute mental stress have primarily been in either animals (Madrigal et al., 2001; Bagchi et al., 1999) or observational studies in humans (Sivonova et al., 2004; Lesgards et al., 2002). For example, medical students undergoing a stressful examination period demonstrated increased DNA and lipid oxidation, as well as a decreased antioxidant capacity when compared to a non-stressful period of their life (Sivonova et al., 2004). Conversely, individuals delivering public speeches showed increased urinary concentrations of bilirubin oxidative metabolite, an abundant antioxidant compound, compared to a control group who did not perform a public speaking task (Yamaguchia et al., 2002). Similar disparity exists with acute mental stress and

ROS production, with studies indicating increased (Bagchi et al., 1999) and suppressed (Atanackovic et al., 2002) production. Collectively these studies warrant careful interpretation due to the different study designs, categorisations of stress, measures of oxidative stress and the time points assessed. To our knowledge only one previous investigation has assessed the acute oxidative stress response to a controlled laboratory mental stress task (Huang et al., 2010). In this study, total antioxidant capacity (TAC) was not perturbed in response to mental stress, however there are potential mechanisms which could explain an increase in oxidative stress in response to mental stress. For example, the well documented ROS burst from inflammatory neutrophils (Babior et al., 1973) could follow from a mental stress-induced inflammatory response (Veldhuijzen van Zanten et al., 2005; Brydon et al., 2005). Furthermore, altered blood flow and shear stress patterns (i.e. oscillatory shear) in response to mental stress (Veldhuijzen van Zanten et al., 2009) could generate ROS via vascular oxidases (Harrison et al., 2003), thus enhancing oxidative stress. Finally, a direct mechanism between oxidative stress and mental stress-induced MI might be explained by the actions of the diverse reactive nitrogen species (RNS), nitric oxide (NO). NO can regulate blood vessel dilation and vascular health (Lewis et al., 1999), however its function may be inhibited by mental stress-induced sequestering of NO by ROS (Beckman, 1996). Despite these potential mechanisms, the sources of oxidative stress and the mechanisms leading to MI as a result of mental stress have yet to be explored in detail.

There is evidence to suggest that ROS can trigger inflammatory pathways in humans (Naik and Dixit, 2011), and inflammation is known to drive ROS production (Babior et al., 1973). Studies in clinical populations with high underlying inflammation have reported an exaggerated inflammatory response to acute mental stress (Kop et al., 2008; Veldhuijzen van Zanten et al., 2008). It should be noted that, due to the underlying factors associated with these clinical populations (e.g., other cardiovascular risk factors), it is impossible to explore

the direct effect of inflammation. As a result, laboratory based models have been used to induce an inflammatory response in healthy subjects (Clapp et al., 2004; Hingorani et al., 2000) and recently to specifically investigate the role of inflammation on mental stress-induced responses (Paine et al., 2013a). These studies have demonstrated that an acute rise in inflammation can influence the vascular responses to mental stress. However, the impact of baseline inflammation on the oxidative stress response to mental stress has yet to be examined.

Eccentric exercise-induced muscle damage and vaccination (*Salmonella typhi* (typhoid) capsular polysaccharide vaccine) have previously been used as models to induce systemic inflammation. In these studies, a peak inflammatory cytokine response was observed 6 hours following both inflammatory stimuli (MacIntyre et al., 2001; Paulsen et al., 2005; Paine et al., 2013b; Antoniadou et al., 2011). The use of a low dose typhoid vaccination to induce a mild systemic inflammatory response is typically safe and approved by the US food and drug agency (Hingorani et al., 2000; Strike et al., 2004; C.E. Wright, P.C. Strike, L. Brydon, 2005). The administration of a mild pathogen can stimulate the adaptive immune system to respond by mobilising white blood cells and increasing the production of inflammatory cytokines. Importantly, it has been demonstrated that a needle injection alone does not cause a significant inflammatory response (Paine et al., 2013b). Similarly, eccentric exercise is commonly used as a safe experimental manipulation to study acute increases in inflammation (Paine et al., 2013a). Elongation of the activated muscle under tension can create a localised inflammatory response within the muscle that can infiltrate the systemic circulation (Nosaka et al., 2002; Proske and Morgan, 2001). In both of these models, the most typically studied inflammatory marker is Interleukin (IL-6), an acute phase protein with an active role in the inflammatory response (Papanicolaou et al., 1998). Importantly, IL-6 is a

commonly used inflammatory marker in response to mental stress in the literature (Steptoe et al., 2007).

To our knowledge no previous studies have explored changes in multiple markers of oxidative stress to acute mental stress following experimental manipulations of baseline inflammation. The aim of the current investigation was to use two inflammatory paradigms (vaccination and eccentric exercise) to assess how plasma markers of oxidative stress were perturbed in response mental stress. It was hypothesised that mental stress would elicit increases in oxidative stress, and that higher baseline levels of inflammation would exaggerate this response.

2.3. Methods

2.3.1. Participants

Vaccination paradigm

Seventeen healthy males were recruited from the University of Birmingham. All participants gave their informed written consent and the vaccination paradigm of the study was approved by NHS Black Country NRES ethics committee. None of the participants had recently suffered from acute illness or infection. No participants reported a history of immune disorders, cardiovascular or other chronic inflammatory diseases and all participants were non-smokers. Participants were asked to refrain from vigorous exercise, alcohol and foods high in dietary nitrate 24 hours prior to testing. Participants were also asked to abstain from food and caffeine two hours beforehand. No participants had received a *Salmonella typhi* capsular polysaccharide vaccine in the preceding year.

Eccentric Exercise paradigm

Seventeen healthy males were recruited from the University of Birmingham. The same exclusion and adherence criteria were used as indicated in the vaccination paradigm. In addition, all participants were unaccustomed to regular eccentric exercise training. As with the vaccination paradigm, participants gave their informed written consent and the eccentric exercise paradigm was approved by the appropriate ethics committee at the University of Birmingham.

2.3.2. Vaccination Protocol

Participants received an intra-muscular injection of *S. typhi* capsular polysaccharide vaccine (0.025mg in 0.5ml, Typhim Vi, Sanofi Pasteur, UK) into the deltoid muscle of the non-dominant arm. A trained nurse administered and then monitored the participant for half

an hour following injection. Participants then completed questionnaires for demographic and medical purposes, as well as questionnaires addressing symptoms associated with the injection.

2.3.3. Eccentric Exercise Protocol

The eccentric exercise protocol was adapted from a previous study (Jackman et al., 2010). Using a Cybex leg extension machine (Cybex International Medway, MA), the 1 repetition maximum (1RM) of participant's non-dominant leg was determined. From a flexed position, participants were required to concentrically lift their leg into an extended position and hold for 2 seconds. 1RM was determined within 5 attempts to avoid fatigue. The 1RM was established when participants could no longer lift or hold the weight for longer than 2 seconds. The eccentric exercise task was then explained to participants before completing a practice set at 50% 1RM to ensure an understanding of the task action. The task required participants to lower the weight (120% 1RM) with their dominant leg from an extended position (15 degrees flexion) to a flexed position (110 degrees flexion) over a 4 second period. Two experimenters lifted the weight into the extended position between repetitions. The protocol required participants to complete 12 sets of 5 repetitions with 5 seconds rest between each repetition and 1 minute rest in-between each set. Incomplete repetitions (i.e. where the weight was lowered in less than 4 seconds) were repeated. Participants then completed questionnaires for demographic and medical background purposes.

2.3.4. Control Protocol

In the eccentric exercise paradigm of the study, participants rested quietly for 25 minutes and then completed questionnaires administered for demographic and medical

background information. No visit was required for subjects in the vaccination paradigm of the study.

2.3.5. Mental Stress Task

A 16-minute version of the paced auditory serial addition task (PASAT) was used to induce acute mental stress in all participants (Veldhuijzen van Zanten et al., 2005). A series of single digit numbers were dictated to participants via a CD player. The objective was to add each number to the previous number, vocalising the response to an accompanying demonstrator who was sat 1 metre away (Gronwall, 1977; Ring et al., 2002). Participants completed two 8 minute tasks, with a 1 minute interval between each task. The first task consisted of four consecutive 2 minute blocks, delivered at inter-stimulus rates of 2.8, 2.4, 2.0 and 1.6 seconds. The second task used inter-stimulus rates of 2.4, 2.0, 1.6 and 1.2 seconds, with the decrease in inter-stimulus rates increasing task difficulty. Additionally, several other experimental manipulations were implemented to enhance the mental stress response (Veldhuijzen van Zanten et al., 2004). The demonstrator checked participant responses against the correct answers, and a loud aversive noise was sounded within each block of 10 numbers upon the first incorrect or late response, or at the end of each block if no incorrect response was given. Participants were videotaped and instructed to focus their attention on a screen showing their face throughout the task. This manipulation was under the false instruction that they were being analysed by independent body language experts. Finally, a leader board was placed within subject eye line to promote competitiveness, and a promise of £10 for the highest study score in their first session and most improved score in session two. These experimental manipulations have been previously shown to increase provocativeness of the task by increasing demands such as time pressure, social evaluation, punishment and reward (Veldhuijzen van Zanten et al., 2004; MacIntyre et al., 2001; Woollard et al., 2002).

2.3.6. Procedure

Participants were subjected to one of the inflammatory paradigms (vaccination or eccentric exercise). All participants conducted the stress reactivity session twice, which were scheduled at least seven days apart: once in an inflammation condition and once in a control condition. Both inflammation conditions involved a morning visit (starting: 08.00-10.00), during which the protocols described above was carried out, and an afternoon (starting 14.00-16.00) visit, during which the stress reactivity session was conducted. (figure 2.1). The six hour time window between the visits was based on previous research that indicates a peak inflammatory cytokine response to typhoid vaccination (Paine et al., 2013b) and eccentric exercise at this time point (MacIntyre et al., 2001). In the morning of the control condition, participants were either asked to attend the laboratory to complete questionnaires (eccentric exercise paradigm) or no visit was needed (vaccination paradigm). At the start of the first reactivity session, participants were asked to complete a diet questionnaire. A copy was then given to each participant and they were asked to repeat their diet before the second reactivity session.

2.3.7. Stress Reactivity Session

Following the assessments of height and weight (*Seca Alpha, Hamburg, Germany*), participants lay in a supine position for the remainder of the session. A small cuff was placed around the middle finger of the dominant hand for continuous recordings of heart rate (HR), systolic (SBP) and diastolic blood pressure (DBP) throughout the session (Finapres Medical Systems; Amsterdam, The Netherlands). Data was recorded via a Power 1401 (CED) connected to a computer with Spike 2 analysis software (version 6). A catheter (Becton, Dickson & Company, Oxford, UK) was inserted into the antecubital vein of the dominant arm. The catheter was kept patent throughout the session with regular saline flushes.

Participants rested for 20 minutes whilst watching a BBC nature documentary, with a blood sample taken (baseline). Following a practice, participants completed the mental stress task as detailed above. A blood sample was taken during the last minute of the second PASAT block (stress). Participants then completed a 30 minute recovery period, which involved participants resting in the supine position whilst watching a BBC nature documentary. A blood sample was taken at the end of this period (post+30). Blood (12 ml per time point) was collected into vacutainers containing potassium ethylene diaminetetraacetic acid, stored on ice until centrifugation (1500g for 10 minutes at 4°C) and plasma aliquoted and stored at -80°C for future analyses.

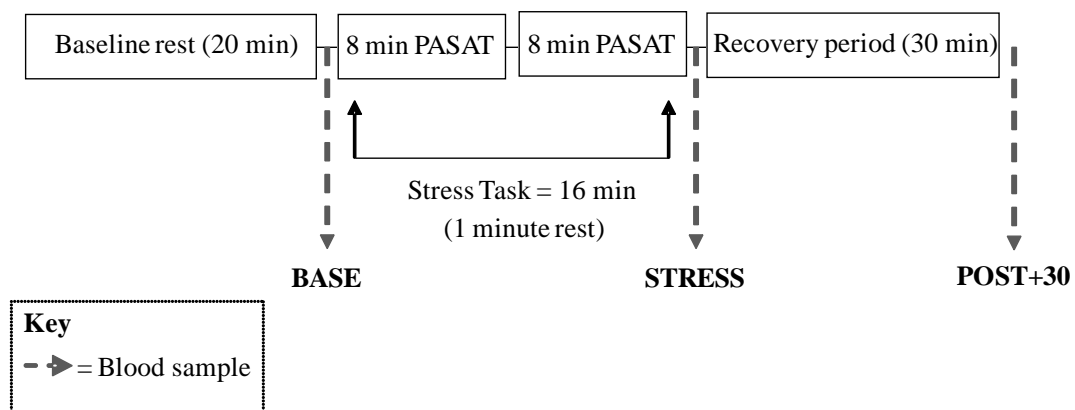


Figure 2.1: Stress Reactivity Session: A schematic representation of the study protocol.

2.3.8. Blood Analyses

Blood samples were assessed for blood cell composition, specifically total white blood cells, granulocytes, monocytes and lymphocytes. Haemoglobin (g/dL) and haematocrit (%) were assessed to calculate plasma volume changes as a result of mental stress (*Coulter Analyser, Beckman-Coulter, High Wycombe, UK*) (Kargotich et al., 1997). All reagents purchased from Sigma unless stated (*Sigma Aldrich, Dorset, UK*).

Total Antioxidant Capacity

TAC was assessed using the Ferric Reducing Ability of Plasma (FRAP) assay (Benzie and Strain, 1996). Plasma samples (10 μ l per well) and standards (ascorbic acid, 0-1000 μ M; 10 μ l) were added in triplicate to a flat bottomed 96 well plate. FRAP reagent (300 mM sodium acetate (pH=3.6), 160 mM 2, 4, 6- tripyridyltriazin and 20mM ferric chloride; 300 μ l) was added to each well and left to incubate for 8 minutes at room temperature, then absorbances read at 650 nm. TAC values were obtained using absorbance values of known ascorbic acid concentrations, expressed as μ M of antioxidant power relative to ascorbic acid (McAnulty et al., 2005) and adjusted for changes in plasma volume.

Protein Carbonylation (PC)

PC was assessed by ELISA (Buss et al., 1997; Carty et al., 2000) in order to quantify the degree of protein oxidation in plasma samples. Samples were diluted in coating buffer (50mM sodium carbonate, pH=9.2) to a concentration of 0.05mg/ml. Samples and standards (50 μ l) were added in triplicate to a 96 well NUNC maxisorb microtitre plate for 1 hour at room temperature. Bound protein was then incubated in the dark for 1 hour at room temperature with 2, 4-dinitrophenylhydrazine (DNPH) (1mM, in 2M HCl). All wells were then blocked (200 μ l) with TBS Tween (0.1%) overnight at 4°C. Wells were incubated with monoclonal mouse anti-DNP antibody (50 μ l, 1:1000) for 2 hours at room temperature, followed by peroxidase conjugated rat anti-mouse IgE conjugated HRP (50 μ l, 1:5000, *AbD Serotec, Oxford, UK*) for 1 hour at room temperature. All steps were followed by three washes to the 96 well plate using TBS Tween (0.05%). Substrate (0.5M Citrate phosphate buffer (10mls, pH=5), hydrogen peroxide (8 μ l) and Ortho-Phenylenediamine tablet (2mg); 50 μ l) was added to each well and the reaction stopped after 20-25 minutes with 2M sulphuric acid (50 μ l). Each well was measured for absorbance at 490nm (Multiscan MS, Labsystems)

and quantified using absorbance values of PC standards made in our lab (1.28 - 5.20 nmols/mg of protein).

Preparation of protein carbonyl standards

Ratios of oxidised (2,2'-Azobis(2-amidinopropane dihydrochloride, 500 mM) to reduced (Sodium Borohydride, 540 mM) bovine serum albumin (BSA, 10 mg/ml in PBS) were prepared and carbonyl content determined as previously described (Buss et al, 1997). Briefly, standards (0-100% oxidation, 2 mg/ml) were incubated in DNPH (10 mM, 500 μ l) or HCL (Control: 2M, 500 μ l) for 1 hour at room temperature with gentle agitation. Protein was precipitated using trichloroacetic acid (20%), vortexed and centrifuged (13,000 rpm, 3 minutes). The supernatant was removed and protein pellet washed three times in ethyl acetate: ethanol solution (1:1), with intermittent centrifugation (13,000 rpm, 1 minute). Standards were then incubated in guanidine hydrochloric acid (37 °C, 60 minutes) and absorbances read at 360 nm. Carbonyl content was determined as nmols/mg protein using an extinction coefficient ($\epsilon_{340} = 24600 \text{ M}^{-1}\text{cm}^{-1}$), and adjusted relative to the absorbance of the control sample.

Protein Concentration

Sample protein concentrations were determined using the bicinchoninic assay method (Smith et al., 1985). Briefly, samples (10 μ l) and standards (BSA; 0-1 mg/ml, 10 μ l) were added to a 96 well microtitre plate in triplicate. Bicinchoninic acid detection reagent (BCA), supplemented with copper II sulphate (20 μ l / ml BCA) was added to each well (200 μ l) and incubated in the dark for 30 minutes at room temperature. Absorbances were read at 540nm, and protein concentrations were determined using absorbance values of known BSA protein concentrations (0 – 1 mg/ml).

Lipid Hydroperoxides

LOOH concentrations were assessed using a spectrophotometric assay (El-Saadani et al., 1989). Plasma samples and a blank standard (10 μ l) were added to a 96 well microtitre plate in triplicate. Reagent mix (100 μ l, 0.2 M Potassium phosphate (pH=6.2), 0.12 M potassium iodide, 0.15 mM sodium azide, Triton X (2 g/l), alkylbenzyldimethylammonium (0.1 g/l), 10 μ M ammonium molybdate and HPLC grade water (to make total volume 100 ml)) was added for 30 minutes at room temperature, away from light on a plate shaker. The plate was read at 340nm (Multiscan MS, Labsystems), concentration of lipid peroxides (μ mol/l) determined using the Beer-Lambert Law (extinction co-efficient $\epsilon_{340} = 24600 \text{ M}^{-1}\text{cm}^{-1}$) and adjusted for changes in plasma volume.

Nitrite and Nitrate (NO_x metabolites)

The Griess assay was used to quantify total nitric oxide metabolites (NO_x) in plasma samples (Miranda et al., 2001; Moshage et al., 1995). Samples (100 μ l) were diluted with HPLC grade water (375 μ l) and zinc sulphate (25 μ l, 300 mg/ml) to give a concentration of approximately 15 mg/ml. Samples were vortexed and then centrifuged at 10,000 g for 20 minutes (10 °C). Supernatants and standards (100 μ l) were added to a 96 well microtitre plate in triplicate, and 100 μ l vanadium (III) chloride (8 mg/ml) then added. Thorough mixing was ensured to fully reduce plasma nitrate to nitrite. Sulphanilamide (50 μ l, 2 %) and N-(1-naphthyl) ethylenediamine dihydrochloride (50 μ l, 0.1 %) were then rapidly added to each well. Plates were incubated for 30 minutes at 37°C, absorbance read at 540nm and compared with values of known nitrite concentrations (0-100 μ M). Values were adjusted for changes in plasma volume.

Interleukin-6

Plasma concentrations of IL-6 were determined using a commercially available High-Sensitivity ELISA kit according to manufacturer instructions (*R&D Systems*). Briefly, samples (200 µl, diluted 1:1 with assay diluent) were incubated with a monoclonal antibody (200 µl) derived against IL-6, and then substrate (50 µl) and amplifier (50 µl) solutions added to develop the colour change. Plates were washed intermittently to reduce non-specific binding. The reaction was stopped using 2 M sulphuric acid (50 µl) and absorbance's read at 650nm. Values were then obtained from a linear standard curve of known IL-6 concentrations (0.156-10 pg/ml) and adjusted for changes in plasma volume.

2.3.9. Data reduction and analysis

Heart rate, SBP and DBP were recorded continuously throughout the stress reactivity session. Data was analysed during minutes 14, 16, 18, and 20 of the baseline rest period, at 2-minute intervals during the stress task and at minutes 24, 26, 28, and 30 of the recovery period. Subsequently, the values were averaged to derive an overall baseline, stress and post+30 value for HR, SBP and DBP. Statistical analyses were performed using SPSS (PASW Statistics, release 18.0, SPSS Inc., Chicago, IL, USA). Differences in baseline IL-6 values were assessed by independent samples t-tests. The effects of mental stress on oxidative stress and cardiovascular activity were assessed by 2 condition (vaccination or eccentric exercise, control) by 3 time (baseline, stress and post+30) repeated measures ANOVA, with Greenhouse-Geisser correction (Vasey and Thasey, 1987). Post hoc analysis of the interaction effects was performed by a test of simple effects by pairwise comparisons, with Bonferroni correction. Pearson correlations were conducted to examine associations between baseline inflammation and oxidative stress with changes in LOOH, PC, TAC or NO_x from

baseline to stress. These analyses were performed separately for control and inflammatory conditions. Statistical significance was accepted at the $p < .05$ level.

2.4. Results

2.4.1. Vaccination paradigm

Vaccination induced inflammation

Table 2.1 describes the characteristics of the participants in the vaccination paradigm of the study. Figure 2.2 outlines IL-6 concentrations at baseline in the control and vaccination conditions. IL-6 concentrations in the vaccination condition were higher than in the control condition ($p < .001$), confirming that vaccination increased inflammation prior to the start of the stress task.

Table 2.1: Characteristics of participants in the vaccination paradigm (Mean \pm standard deviation)

Participant Characteristics	(N=17)
Age (yrs)	20 (1)
Weight (kg)	81.3 (9.4)
Height (m)	1.8 (0.1)
BMI (kg/m²)	24.5 (2.2)

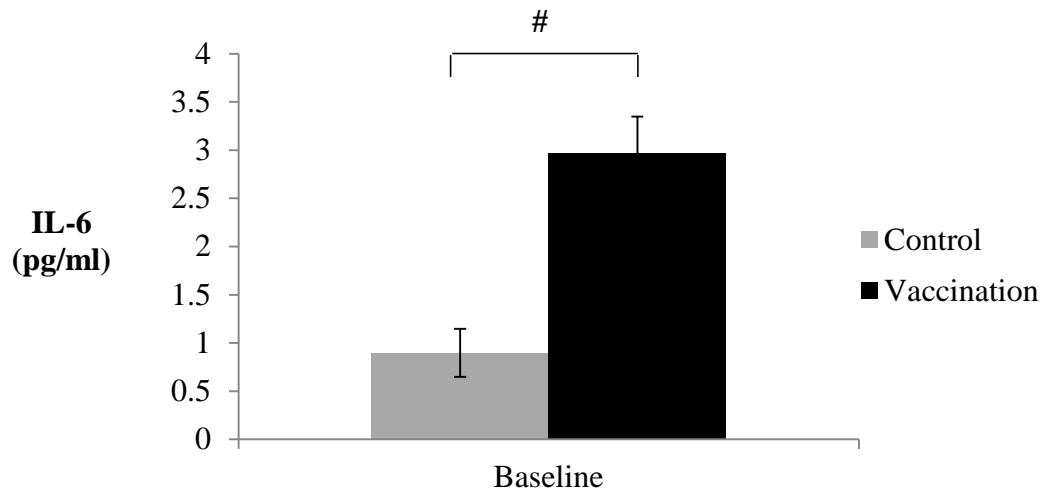


Figure 2.2: IL-6 concentrations at baseline in control and vaccination conditions. # indicates a significant difference between control and vaccination conditions ($p < .05$)

White blood cell subset responses

The effects of mental stress on white blood cell subsets in the vaccination paradigm are shown in Table 2.2. Total white blood cell and granulocyte numbers were higher in the vaccination condition relative to the control condition (condition effects; p 's $< .001$). Total white blood cell, granulocyte and lymphocyte numbers increased, and monocyte number decreased in response to mental stress in both conditions (time effects: $p < .026$). Pairwise comparisons revealed that lymphocyte number increased during stress, relative to baseline in both conditions (time effect: $p = .001$) and decreased, relative to stress post+30 (time effect: $p = .027$). There was a significant condition * time interaction effect for total white blood cell number. Post hoc analyses revealed that total white blood cell number was elevated during stress in both conditions, but remained elevated post+30, relative to baseline in the control condition only.

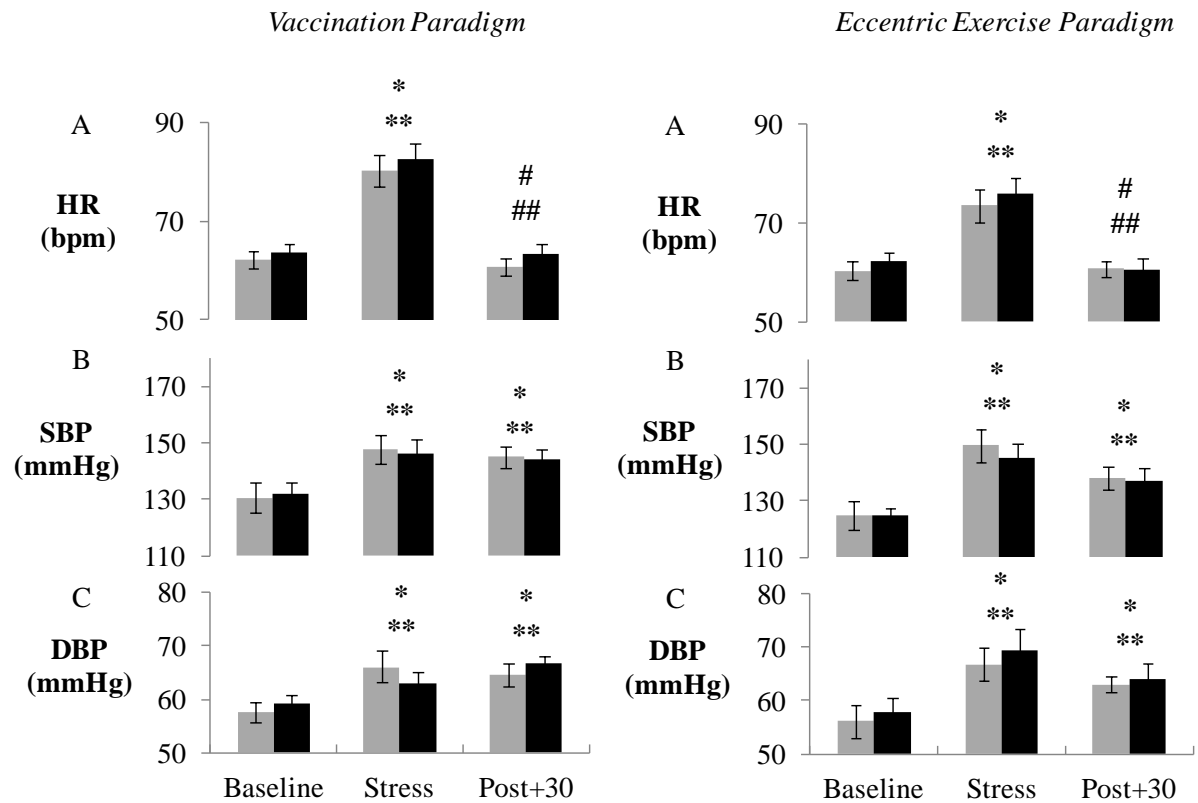
Table 2.2: White blood cell subset concentrations (mean \pm standard deviation) at baseline, stress and post+30 in the control and vaccination conditions

	Control Condition			Vaccination Condition			Time effect	Condition effect	Time * condition effect
	Baseline	Stress	Post+30	Baseline	Stress	Post+30			
White Blood Cells ($\times 10^9/L$)	5.98 \pm 0.28	6.43 \pm 0.27*	6.76 \pm 0.29*	9.32 \pm 0.35	9.69 \pm 0.29*	9.53 \pm 0.27	p<.001	p<.001	p=.024
Granulocytes ($\times 10^9/L$)	3.96 \pm 0.29	4.23 \pm 0.27	4.82 \pm 0.30	6.94 \pm 0.30	6.79 \pm 0.46	7.87 \pm 0.58	p=.026	p<.001	p=.597
Monocytes ($\times 10^9/L$)	0.39 \pm 0.17	0.36 \pm 0.12	0.27 \pm 0.10	0.43 \pm 0.19	0.43 \pm 0.18	0.35 \pm 0.19	p=.026	p=.098	p=.772
Lymphocytes ($\times 10^9/L$)	1.76 \pm 0.31	1.94 \pm 0.31*	1.79 \pm 0.26#	1.88 \pm 0.28	2.01 \pm 0.27*	1.91 \pm 0.35#	p=.002	p=0.096	p=.605

* indicates significant differences relative to baseline ($p<.05$). # indicates significant differences relative to stress ($p<.05$).

Cardiovascular responses

Figure 2.3 (left panel) outlines the cardiovascular responses to the mental stress task in control and vaccination conditions. HR increased over the course of the mental stress task and returned to baseline values in both conditions (time effects; $p < .001$). SBP and DBP increased over the course of the stress task and remained elevated above baseline values post+30 (time effects; p 's $< .05$). No differences were observed in HR, SBP and DBP between conditions.



*Figure 2.3: Cardiovascular responses to mental stress. The changes in heart rate (A), systolic blood pressure (B) and diastolic blood pressure (C) at baseline, during and 30 minutes following the mental stress task in both the control (grey bars) and inflammation (black bars) condition in the vaccination paradigm (left panel) and the eccentric exercise paradigm (right panel). Data are means \pm standard error. * indicates significant differences relative to baseline in the control condition ($p < .05$). ** indicates significant differences relative to baseline in the inflammation condition ($p < .05$). # indicates significant differences relative to stress in the control condition ($p < .05$). ## indicates significant differences relative to stress in the inflammation condition ($p < .05$)*

Oxidative stress responses

Figure 2.4 (left panel) outlines the changes in markers of oxidative stress and nitric oxide metabolites in response to the mental stress task in control and vaccination conditions.

PC and NOx values were higher in the vaccination condition than the control condition (condition effects; p 's <.05). TAC, PC, LOOH and NOx were unaltered in response to mental stress in both conditions (time effects: p 's > .126).

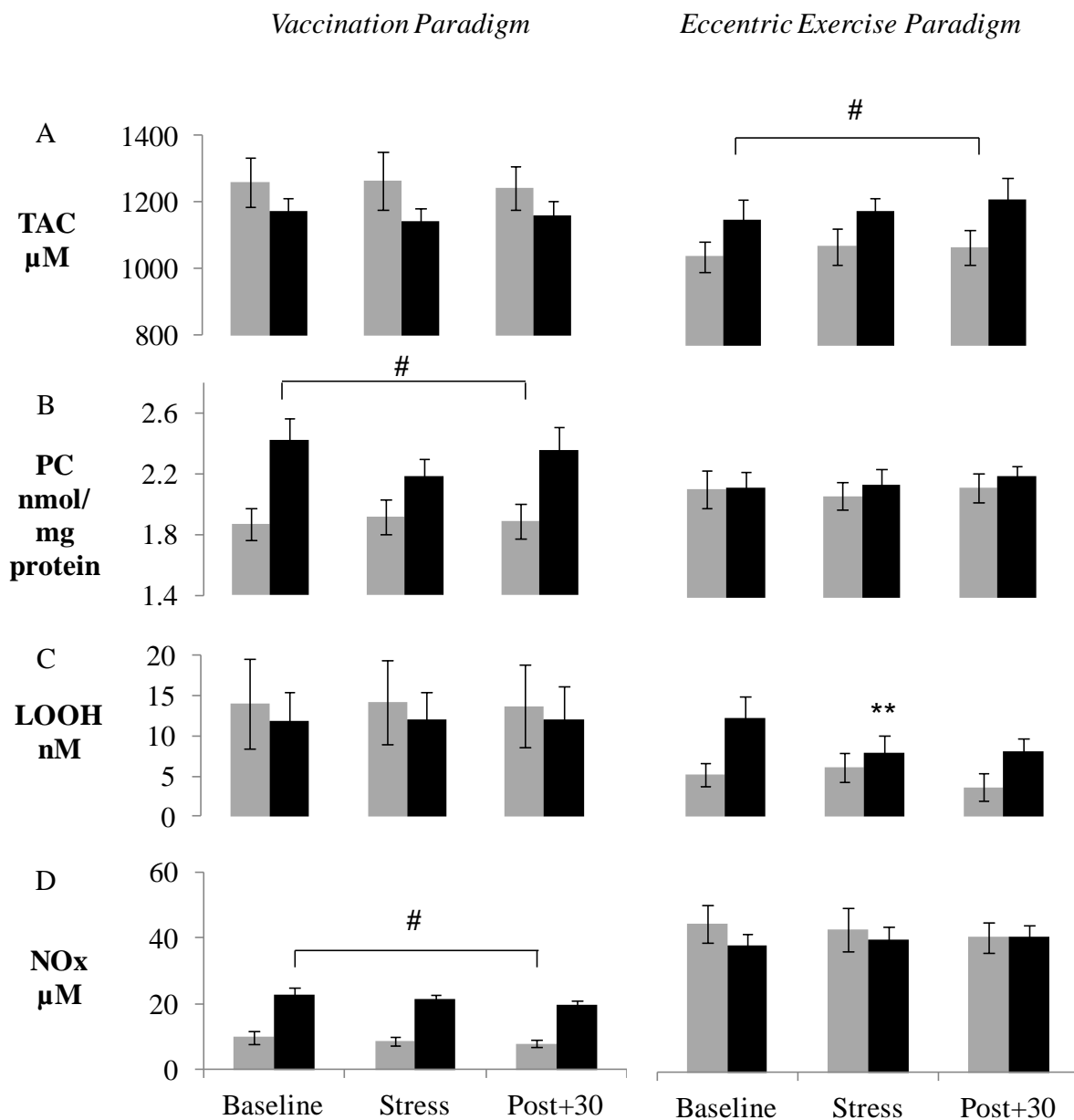


Figure 2.4: Oxidative stress responses to mental stress. The changes in TAC (A), PC (B), LOOH (C) & NOx (D) at baseline, stress and 30 minutes following the mental stress task in

both the control (grey bars) and inflammation (black bars) condition in the vaccination paradigm (left panel) and the eccentric exercise paradigm (right panel). Data are means \pm standard error. ** indicates significant changes relative to baseline ($p < .05$). # indicates a significant difference between control and inflammatory conditions ($p < .05$).

Associations between baseline oxidative stress and inflammation and oxidative stress responses to mental stress

No associations were observed when assessing the influence of baseline markers of oxidative stress and inflammation on the LOOH, PC, TAC or NOx response to mental stress in control or vaccination conditions.

2.4.2. Eccentric exercise paradigm

Eccentric exercise induced inflammation

Table 2.3 describes the characteristics of the participants in the eccentric exercise paradigm of the study. Figure 2.5 outlines IL-6 concentrations in the control and eccentric exercise conditions. IL-6 concentrations in the eccentric exercise condition were higher than in the control condition ($p = .01$), confirming that eccentric exercise had increased inflammation prior to the start of the stress task.

Table 2.3: Characteristics of participants in the eccentric exercise paradigm (Mean \pm standard deviation)

Participant Characteristics	(N=17)
Age (yrs.)	21 (1)
Weight (kg)	76.6 (8.8)
Height (m)	1.8 (0.1)
BMI (kg.m ²)	23.6 (2.4)

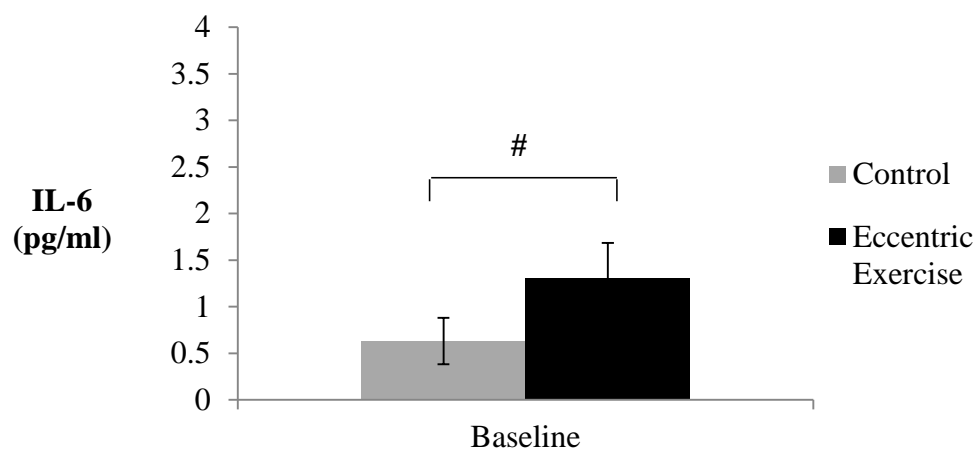


Figure 2.5: IL-6 concentrations at baseline in control and eccentric exercise conditions. # indicates a significant difference between control and vaccination conditions ($p < .05$)

White blood cell subset responses

The effect of mental stress on white blood cell subsets in the eccentric exercise paradigm is shown in Table 2.4. Total white blood cell number increased during stress in both conditions ($p = .007$), with no differences observed between conditions. No differences in lymphocyte, monocyte or granulocyte numbers were observed in response to mental stress

or between conditions. There were no condition * time interaction effects for the white blood cell subsets.

Table 2.4: White blood cell subset concentrations (mean \pm standard deviation) at baseline, stress and post+30 in the control and eccentric exercise conditions

	Control Condition			Eccentric Exercise Condition			Time effect	Condition effect	Time * Condition effect
	Baseline	Stress	Post+30	Baseline	Stress	Post+30			
White Blood Cells ($\times 10^9/L$)	6.03 \pm 0.17	6.44 \pm 0.26*	6.32 \pm 0.32	6.34 \pm 0.37	6.76 \pm 0.42*	6.61 \pm 0.41	p=.007	p=.293	p=.937
Granulocytes ($\times 10^9/L$)	3.77 \pm 0.87	3.81 \pm 1.17	3.72 \pm 1.12	4.14 \pm 1.27	4.31 \pm 1.47	4.23 \pm 1.37	p=.315	p=.102	p=.549
Monocytes ($\times 10^9/L$)	0.30 \pm 0.33	0.27 \pm 0.33	0.43 \pm 0.39	0.38 \pm 0.21	0.43 \pm 0.29	0.50 \pm 0.38	p=.115	p=.297	p=.678
Lymphocytes ($\times 10^9/L$)	2.03 \pm 0.35	2.18 \pm 0.39	2.30 \pm 0.81	1.99 \pm 0.65	2.04 \pm 0.41	1.88 \pm 0.32	p=.607	p=.106	p=.154

* indicates significant differences in baseline values relative to the control conditions ($p < .05$).

indicates significant differences in values relative to stress ($p < .05$).

Cardiovascular responses

Figure 2.3 (right panel) outlines the cardiovascular responses to the mental stress task in control and eccentric exercise conditions. Heart rate increased immediately following the mental stress task and returned to baseline values in both conditions (time effect; $p < .001$). SBP and DBP increased immediately following the mental stress task and remained elevated above baseline values post+30 (time effects; p 's $< .05$). No differences were observed in HR, SBP and DBP between conditions.

Oxidative stress responses

Figure 2.4 (right panel) outlines the changes in markers of oxidative stress and nitric oxide metabolites in response to the mental stress task in control and eccentric exercise conditions. TAC values were higher in the inflammation condition compared to the control condition (condition effect; $p = .011$). TAC, PC and NOx were unaltered in response to mental stress in both conditions. LOOH decreased in response to mental stress in the eccentric exercise condition only (time effect; $p < .05$).

Associations between baseline oxidative stress and inflammation and oxidative stress responses to mental stress

An association was found between baseline LOOH values and changes in LOOH between baseline and stress in the eccentric exercise condition only ($r = -0.635$, $p < .003$). No other associations were observed when assessing the influence of baseline markers of oxidative stress and inflammation on the PC, TAC or NOx response to mental stress in control or eccentric exercise conditions.

Discussion

The current study demonstrates that vaccination and eccentric exercise were successful models of inducing inflammation, as demonstrated by significantly higher IL-6 concentrations in the inflammation conditions compared to the control conditions (figures 2.2 & 2.5). Nevertheless, acute inflammation did not influence the oxidative stress response to mental stress in either paradigm of this study.

The oxidative stress response to mental stress has previously been unclear, either under conditions of high or basal inflammation. Previous studies have indicated an increase (Sivonova et al., 2004; Bagchi et al., 1999), decrease (Atanackovic et al., 2002; Yamaguchia et al., 2002) and no change (Huang et al., 2010) in indices of oxidative stress (i.e. plasma markers and ROS levels) in response to acute mental stress and during periods of lifestyle stress. Oxidation of macromolecules within the body is part of normal homeostatic processes that regulate cellular signalling, growth and adaptations within tissues. Many lifestyle factors, including health history, nutritional status (Møller et al., 1996) and even internal circadian rhythm (Kanabrocki et al., 2002) can influence the degree of oxidative modification to these macromolecules. As a result, previous observational studies monitoring the impact of mental stress on markers of oxidative stress/ROS at one 'snap shot' warrant careful interpretation. In addition, stressful situations (i.e. exam periods (Sivonova et al., 2004) and public speaking (Yamaguchia et al., 2002)) and study model (human (Atanackovic et al., 2002) and animal (Bagchi et al., 1999) models) have varied significantly between studies, making it hard to draw valid and robust conclusions. To our knowledge, this was the first study utilising well standardised laboratory models of both acute inflammation and mental stress to test the sole influence of baseline inflammation on the oxidative stress responses to mental stress in humans. Our findings show that mental stress did not increase PC, LOOH, TAC and NOx under conditions of basal or experimentally manipulated elevated inflammation (figure 2.4).

Previous studies have suggested a role for baseline inflammation on physiological responses to mental stress (Veldhuijzen van Zanten et al., 2005; Shah et al., 2006; Roupe van der Voort et al., 2000). In the current study, we used two established experimental paradigms to induce inflammation in healthy participants to specifically assess the influence of inflammation on the oxidative stress responses to mental stress. The elevations in baseline IL-6 concentrations in both of the inflammatory conditions, relative to control were comparable to values observed in patients with coronary artery disease (Kop et al., 2008) and otherwise apparently healthy men who then suffered a MI (Ridker et al., 2000). Thus, the experimental manipulations induced increase in IL-6 values which were similar to those at risk for MI. Furthermore, the mental stress task used in the present study elicited a physiological response similar to previous studies (Veldhuijzen van Zanten et al., 2004; Paine et al., 2013a; Veldhuijzen van Zanten et al., 2005). Heart rate and blood pressure (figure 2.3) and total number of white blood cells (tables 2.2 and 2.4) increased in all conditions during the mental stress task. As a result we cannot attribute the lack of change in markers of oxidative stress to the models of inflammation or mental stress utilised in the current study.

The potential sources of ROS in response to mental stress have not been previously clarified. Respiratory bursts from phagocytes (Babior et al., 1973) and increased oscillatory shear stress (Harrison et al., 2003) have been identified as two possible sources. Interestingly inflammation is known to increase the amount of circulating phagocytic cells and alter blood flow patterns (Nagel et al., 1994), therefore providing a clear rationale for increased oxidative stress in response to mental stress. The current data indicate that markers of oxidative stress and nitric oxide metabolism were elevated at baseline in the inflammatory conditions only (figure 2.4), indicating that inflammation had induced oxidative stress and NO production, however the same markers remained stable in response to mental stress. Interestingly, despite white blood cell numbers being higher at baseline in the inflammatory conditions relative to

control, this did not exacerbate any white blood cell responses to mental stress (table 2.2). Given the role of respiratory bursts on RONS production, the similarity in white blood cell responses in control and inflammatory conditions may explain, in part, the similar responses of markers of oxidative stress.

It must be noted that a decrease in LOOH was observed in the inflammation condition in the eccentric exercise paradigm only (time effect: $p < .05$) (figure 2.4C, right panel). Further investigation revealed that the decrease in LOOH from baseline to post-stress was associated with higher baseline LOOH values ($r = -0.635$, $p < .003$). Thus, this could suggest that elevated levels of oxidised lipids may have been removed in response to mental stress, rather than a reduction in lipid oxidation *per se*. However, this is speculation and warrants further investigation.

Given the complexity of redox events *in vivo*, it must be considered that specific, rather than global oxidative events may be occurring in response to mental stress. Oxidative adducts, i.e. protein and lipid modifications or altered antioxidant status are indirect markers of RONS-mediated actions. These markers are a good representation of non-cellular, global oxidative change. Further investigation is needed to monitor intracellular markers of oxidative stress, and cellular redox status in response to mental stress. Indeed, specific, rather than global oxidative changes have been observed in response to physiological stressors, such as exercise (Aldred and Rohalu, 2011). In addition, extensive time point analysis is required to account for potential ‘delayed’ responses to mental stress. Some previous studies have reported an increase in IL-6 within thirty minutes of stress (Burns et al., 2008; Edwards et al., 2006). However, other studies have reported increases in markers of inflammation up to 120 minutes following acute mental stress, with no differences observed at 30 minutes, relative to baseline (Brydon et al., 2004; Steptoe et al., 2001). Furthermore, physiological stimuli such

vaccination (Clapp et al., 2004) and exercise (Michailidis et al., 2007) have been shown to elicit delayed increases in markers of oxidative stress.

It must be noted that only selective markers of oxidative stress were measured in the current study. Given the complexity of oxidative events *in vivo*, other plasma and indeed intracellular oxidative changes in response to mental stress cannot be dismissed. In addition, the measurement of leukocyte sub-set numbers and IL-6 as sole indicators of inflammation might be considered a limitation. However, IL-6 is an integral signalling cytokine molecule in the acute phase response, regulating the upregulation of numerous other inflammatory proteins (i.e. C-reactive protein, tumour-necrosis factor alpha and IL-1 and IL-10) and therefore a good representation of an inflammatory response (Febbraio et al., 2010). In addition, IL-6 is a known, robust responder to both vaccination (Paine et al., 2013b) and eccentric exercise (MacIntyre et al., 2001), peaking similarly at 6 hours.

The current data provide evidence that experimentally manipulated baseline inflammation does not increase plasma oxidative stress responses to acute mental stress within thirty minutes in young and healthy males.

2.5. Reference list for chapter two

Aldred, S. and Rohalu, M. (2011) A moderate intensity exercise program did not increase the oxidative stress in older adults. **Arch Gerontol Geriatr**, 53 (3): 350–353

Antoniades, C., Cunningham, C., Antonopoulos, A., et al. (2011) Induction of vascular GTP-cyclohydrolase I and endogenous tetrahydrobiopterin synthesis protect against inflammation-induced endothelial dysfunction in human atherosclerosis. **Circulation**, 124 (17): 1860–70

Atanackovic, D., Djordje Atanackovic, M.D., M. C. Brunner-Weinzierl, H.K., et al. (2002) Acute psychological stress simultaneously alters hormone levels, recruitment of lymphocyte subsets and production of reactive oxygen species. **Immunology Investigations**, 21 (2): 73–91

Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. **Journal of Clinical Investigation**, 52 (3): 741–744

Babyak, M.A., Blumenthal, J.A., Hinderliter, A., et al. (2011) Prognosis after change in left ventricular ejection fraction during mental stress testing in patients with stable coronary artery disease. **American Journal of Cardiology**, 105 (1): 25–28

Bagchi, D., Carryl, O.R., Tran, M.X.M., et al. (1999) Acute and chronic stress-induced oxidative gastrointestinal mucosal injury in rats and protection by bismuth subsalicylate. **Molecular and Cellular Biochemistry**, 31: 109–116

Beckman, J.S. (1996) Oxidative damage and tyrosine nitration from peroxynitrite. **Chemical Research in Toxicology**, 9 (5): 836–844

Benzie, I.F. and Strain, J.J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. **Analytical Biochemistry**, 239 (1): 70–76

Bergovec, M., Sjerobabski, V., Farrar, G., et al. (1992) Acute myocardial infarction among civilians in Zagreb city area. **Lancet**, 339 (8788): 303

Brydon, L., Edwards, S., Jia, H., et al. (2005) Psychological stress activates interleukin-1 β gene expression in human mononuclear cells. **Brain, behavior, and immunity**, 19 (6): 540–6

Brydon, L., Edwards, S., Mohamed-Ali, V., et al. (2004) Socioeconomic status and stress-induced increases in interleukin-6. **Brain, Behavior, and Immunity**, 18 (3): 281–290

Burns, V.E., Edwards, K.M., Ring, C., et al. (2008) Complement cascade activation after an acute psychological stress task. **Psychosomatic Medicine**, 70 (4): 387–396

- Buss, H., Chan, T.P., Sluis, K.B., et al. (1997) Protein carbonyl measurement by a sensitive ELISA method. **Free Radical Biology and Medicine**, 23 (3): 361–366
- Wright, C.E., Strike, P.E., Brydon, L., et al. (2005) Acute inflammation and negative mood: Mediation by cytokine activation. **Brain Behavior and Immunity**, 19 (4): 345–350
- Cai, H. and Harrison, D.G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. **Circ Res**, 87 (10): 840–844
- Carty, J.L., Bevan, R., Waller, H., et al. (2000) The effects of vitamin C supplementation on protein oxidation in healthy volunteers. **Biochem Biophys Res Commun**, 273 (2): 729–735
- Clapp, B.R., Hingorani, A.D., Kharbanda, R.K., et al. (2004) Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress. **Cardiovasc Res**, 64 (1): 172–178
- Dhalla, N.S., Temsah, R.M. and Netticadan, T. (2000) Role of oxidative stress in cardiovascular diseases. **Journal of Hypertension**, 18 (6): 655–673
- Edwards, K.M., Burns, V.E., Ring, C., et al. (2006) Sex differences in the interleukin-6 response to acute psychological stress. **Biological psychology**, 71 (3): 236–9
- El-Saadani, M., Esterbauer, H., El-Sayed, M., et al. (1989) A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. **Journal of Lipid Research**, 30 (4): 627–630
- Febbraio, M.A., Rose-John, S. and Pedersen, B.K. (2010) Is interleukin-6 receptor blockade the Holy Grail for inflammatory diseases? **Clinical pharmacology and therapeutics**, 87 (4): 396–8
- Gronwall, D.M. (1977) Paced auditory serial-addition task: a measure of recovery from concussion. **Perception Motor Skills**, 44: 367–373
- Harrison, D., Griendling, K.K., Landmesser, U., et al. (2003) Role of oxidative stress in atherosclerosis. **Am J Cardiol**, 91 (3A): 7A–11A
- Hingorani, A.D., Cross, J., Kharbanda, R.K., et al. (2000) Acute Systemic Inflammation Impairs Endothelium-Dependent Dilatation in Humans. **Circulation**, 102 (9): 994–999
- Huang, C.J.J., Franco, R.L., Evans, R.K., et al. (2010) Effects of mental challenge on neurovascular responses in healthy male subjects. **Int J Psychophysiol**, 78 (3): 225–230
- Jackman, S.R., Witard, O.C., Jeukendrup, A.E., et al. (2010) Branched-chain amino acid ingestion can ameliorate soreness from eccentric exercise. **Medicine and science in sports and exercise**, 42 (5): 962–70

- Kanabrocki, E.L., Murray, D., Hermida, R.C., et al. (2002) Circadian variation in oxidative stress markers in healthy and type II diabetic men. **Chronobiology International**, 19 (2): 423–439
- Kargotich, S., Goodman, C., Keast, D., et al. (1997) The influence of exercise-induced plasma volume changes on the interpretation of biochemical parameters used for monitoring exercise, training and sport. **Sports medicine**, 7 (3): 185–191
- Kop, W.J., Weissman, N.J., Zhu, J., et al. (2008) Effects of acute mental stress and exercise on inflammatory markers in patients with coronary artery disease and healthy controls. **The American journal of cardiology**, 101 (6): 767–73
- Krantz, D.S., Santiago, H.T., Kop, W.J., et al. (1999) Prognostic value of mental stress testing in coronary artery disease. **American Journal of Cardiology**, 84 (11): 1292–1297
- Lesgards, J.F., Durand, P., Lassarre, M., et al. (2002) Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. **Environmental health perspectives**, 110 (5): 479–86
- Lewis, T.V., Dart, A.M., Chin-Dusting, J.P., et al. (1999) Exercise training increases basal nitric oxide production from the forearm in hypercholesterolemic patients. **Arterioscler Thromb Vasc Biol**, 19 (11): 2782–2787
- MacIntyre, D.L., Sorichter, S., Mair, J., et al. (2001) Markers of inflammation and myofibrillar proteins following eccentric exercise in humans. **European journal of applied physiology**, 84 (3): 180–6
- Madrigal, J.L., Olivenza, R., Moro, M.A., et al. (2001) Glutathione depletion, lipid peroxidation and mitochondrial dysfunction are induced by chronic stress in rat brain. **Neuropsychopharmacology**, 24 (4): 420–429
- McAnulty, S.R., McAnulty, L.S., Morrow, J.D., et al. (2005) Effect of daily fruit ingestion on angiotensin converting enzyme activity, blood pressure, and oxidative stress in chronic smokers. **Free Radic Res**, 39 (11): 1241–1248
- Michailidis, Y., Jamurtas, A.Z., Nikolaidis, M.G., et al. (2007) Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress. **Medicine and science in sports and exercise**, 39 (7): 1107–13
- Miranda, K.M., Espey, M.G. and Wink, D.A. (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. **Nitric Oxide**, 5 (1): 62–71
- Møller, P., Wallin, H. and Knudsen, L.E. (1996) Oxidative stress associated with exercise, psychological stress and life-style factors. **Chemico-biological interactions**, 102 (1): 17–36

- Moshage, H., Kok, B., Huizenga, J.R., et al. (1995) Nitrite and nitrate determinations in plasma: a critical evaluation. **Clin Chem**, 41 (6 Pt 1): 892–896
- Nagel, T., Resnick, N., Atkinson, W.J., et al. (1994) Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. **J Clin Invest.**, 94 (2): 885–891
- Naik, E. and Dixit, V.M. (2011) Mitochondrial reactive oxygen species drive proinflammatory cytokine production. **The Journal of experimental medicine**, 208 (3): 417–20
- Nosaka, K., Newton, M. and Sacco, P. (2002) Muscle damage and soreness after endurance exercise of the elbow flexors. **Med Sci Sports Exerc**, 34 (6): 920–927
- Paine, N.J., Ring, C., Aldred, S., et al. (2013a) Eccentric-exercise induced inflammation attenuates the vascular responses to mental stress. **Brain, behavior, and immunity**, 30: 133–42
- Paine, N.J., Ring, C., Bosch, J.A., et al. (2013b) The time course of the inflammatory response to the Salmonella typhi vaccination. **Brain, behavior, and immunity**, 30: 73–9
- Paine, N.J. and Veldhuijzen van Zanten, J.J.C.S. (2012) Inflammation and vascular responses to acute mental stress: implications for the triggering of myocardial infarction. **Curr. Pharm. Des.**, 18: 1494–1501
- Papanicolaou, D.A., Wilder, R.L., Manolagas, S.C., et al. (1998) The pathophysiologic roles of interleukin-6 in human disease. **Annals of Internal Medicine**, 128 (2): 127–137
- Paulsen, G., Benestad, H.B., StroM-Gundersen, I., et al. (2005) Delayed Leukocytosis and Cytokine Response to High-Force Eccentric Exercise. **Medicine & Science in Sports & Exercise**, 37 (11): 1877–1883
- Proske, U. and Morgan, D.L. (2001) Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. **The Journal of physiology**, 537 (Pt 2): 333–45
- Ridker, P.M., Rifai, N., Stampfer, M.J., et al. (2000) **Plasma Concentration of Interleukin-6 and the Risk of Future Myocardial Infarction Among Apparently Healthy Men.**, 101: 1767–1772
- Ring, C., Drayson, M., Walkey, D.G., et al. (2002) Secretory immunoglobulin A reactions to prolonged mental arithmetic stress: inter-session and intra-session reliability. **Biol Psychol**, 59 (1): 1–13
- Ross, R. (1999) Atherosclerosis--an inflammatory disease. **N Engl J Med**, 340 (2): 115–126

Roupe van der Voort, C., Heijnen, C.J., Wulffraat, N., et al. (2000) Stress induces increases in IL-6 production by leucocytes of patients with the chronic inflammatory disease juvenile rheumatoid arthritis: a putative role for alpha(1)-adrenergic receptors. **Journal of neuroimmunology**, 110 (1-2): 223–9

Shah, R., Burg, M.M., Vashist, A., et al. (2006) C-reactive Protein and Vulnerability to Mental-Stress-Induced Myocardial Ischemia. **Molecular Medicine**, 12 (December): 269–274

Sivonova, M., Zitnanova, I., Hlincikova, L., et al. (2004) Oxidative stress in university students during examinations. **Stress**, 7 (3): 183–188

Smith, P.K., Krohn, R.I., Hermanson, G.T., et al. (1985) Measurement of protein using bicinchoninic acid. **Analytical Biochemistry**, 150 (1): 76–85

Step toe, A., Hamer, M. and Chida, Y. (2007) The effects of acute psychological stress on circulating inflammatory factors in humans: a review and meta-analysis. **Brain Behavior and Immunity**, 21 (7): 901–912

Step toe, A., Willemsen, G., Owen, N., et al. (2001) Acute mental stress elicits delayed increases in circulating inflammatory cytokine levels. **Clin Sci (Lond)**, 101 (2): 185–192

Strike, P.C. and Step toe, A. (2005) Behavioral and emotional triggers of acute coronary syndromes: a systematic review and critique. **Psychosomatic medicine**, 67 (2): 179–86

Strike, P.C., Wardle, J. and Step toe, A. (2004) Mild acute inflammatory stimulation induces transient negative mood. **Journal of Psychosomatic Research**, 57 (2)

Vasey, M.. and Thasey, J.. (1987) The continuing problem of false positives in repeated measures ANOVA in psychophysiology - A multivariate solution. **Psychophysiology**, 24: 479–486

Veldhuijzen van Zanten, J.J., Ring, C., Carroll, D., et al. (2009) Hemoconcentration during a prolonged stress task: associations with hemodynamic reactivity and microvascular permeability. **Biol Psychol**, 82 (3): 260–266

Veldhuijzen van Zanten, J.J.C.S., Kitas, G.D., Carroll, D., et al. (2008) Increase in systemic vascular resistance during acute mental stress in patients with rheumatoid arthritis with high-grade systemic inflammation. **Biol Psychol**, 77 (1): 106–110

Veldhuijzen van Zanten, J.J.C.S., Ring, C., Burns, V.E., et al. (2004) Mental stress-induced hemoconcentration: sex differences and mechanisms. **Psychophysiology**, 42: 98–107

Veldhuijzen van Zanten, J.J.C.S., Ring, C., Carroll, D., et al. (2005) Increased C reactive protein in response to acute stress in patients with rheumatoid arthritis. **Ann Rheum Dis**, 64 (9): 1299–1304

Wadley, A.J., Veldhuijzen van Zanten, J.J.C.S. and Aldred, S. (2012) The interactions of oxidative stress and inflammation with vascular dysfunction in ageing : the vascular health triad. **AGE**, 35 (3): 705–718

Wilbert-Lampen, U., Nickel, T., David, L., et al. (2010) Modified Serum Profiles of Inflammatory and Vasoconstrictive Factors in Patients With Emotional Stress-Induced Acute Coronary Syndrome During World Cup Soccer 2006. **Journal of the American College of Cardiology**, 57 (7): 637–642

Woollard, K.J., Phillips, D.C. and Griffiths, H.R. (2002) Direct modulatory effect of C-reactive protein on primary human monocyte adhesion to human endothelial cells. **Clin Exp Immunol**, 130 (2): 256–262

Yamaguchia, T., Shiojib, I., Sugimotoc, A., et al. (2002) Psychological stress increases bilirubin metabolites in human urine. **Biochemical and Biophysical Research Communications**, 293 (1): 517–520

Chapter Three

3. Three months of moderate intensity exercise reduced 3-nitrotyrosine levels in rheumatoid arthritis patients

3.1. Abstract

Purpose

Rheumatoid arthritis (RA) patients display high levels of oxidative stress. Transient exercise-induced increases in oxidative stress are thought to be adaptive in healthy populations. This study investigated the effect of exercise on markers of oxidative stress in RA, following acute exercise and a period of exercise training.

Methods

Acute exercise study: RA patients (N=12, age: 56 ± 11) undertook a bout of exercise (30-40 min, 70% $\text{VO}_{2\text{MAX}}$) and blood samples were taken before and after exercise to assess markers of oxidative stress. *Training study:* RA patients (N=19, age: 56 ± 10) were randomised into either a control or exercise group, who undertook 3 exercise sessions per week (30-40 min, 70% $\text{VO}_{2\text{MAX}}$) for 3 months. Plasma markers of oxidative stress (protein carbonyls (PC), lipid hydroperoxides (LOOH), 3-nitrotyrosine (3-NT), total antioxidant capacity (TAC) and catalase (CAT) activity), inflammation (interleukin-8 (IL-8) and C-reactive protein (CRP)) and nitric oxide metabolites (NOx) were assessed before and after training.

Results *Acute exercise study:* PC (+18%) and NOx (+27%) were significantly increased following exercise. *Training study:* 3-NT decreased ($2.18 \pm 1.78\mu\text{M}$ to $1.10 \pm 0.93\mu\text{M}$) in the exercise group only, alongside increases in aerobic fitness (24.45 ± 4.98 to 27.10 ± 4.51 ml/kg/min⁻¹) and reductions in disease activity score (DAS: 3.47 ± 1.17 to 2.88 ± 0.76). PC, LOOH, TAC, IL-8, CRP and NOx concentrations, and CAT activity were unchanged in both groups.

Conclusions Aerobic exercise training did not increase markers of oxidative stress in RA patients. 3-nitrotyrosine and disease activity were decreased following exercise training.

3.2. Introduction

Rheumatoid Arthritis (RA) is a chronic systemic inflammatory disease, which is characterised by stiffness, swelling and progressive destruction of joints (Lee and Weinblatt, 2001). In addition, patients with RA have an increased risk for developing cardiovascular disease (CVD) (John et al., 2009; Kitas and Gabriel, 2011). The high levels of inflammation in RA (indicated by interleukins (IL) and C-reactive protein (CRP)) may induce oxidative stress, a state characterised by an imbalance between the production of reactive oxygen and nitrogen species (RONS) and antioxidants. In addition, there is evidence implicating oxidative stress with both joint destruction (Mapp et al., 1995; Winrow et al., 1993; Wink et al., 1996) and elevated CVD risk (Griffiths et al., 2006) in RA.

It has been suggested that oxidative stress and inflammation can reciprocally regulate one another (Wadley et al., 2012), with some evidence for this in RA (Vasanthi et al., 2009; Miyata et al., 1998). Mediators such as IL-8 can initiate respiratory bursts of RONS from inflammatory cells (Babior et al., 1973) and RONS can signal to upregulate IL-8 protein expression (Fu et al., 2002). This reciprocal relationship may exacerbate joint destruction and CVD risk further in RA.

Elevated levels of nitric oxide (NO) (Farrell et al., 1992) and superoxide (O_2^-) (Biemond et al., 1986) in RA may form peroxynitrite ($ONOO^-$). $ONOO^-$ is a RONS capable of nitrating amino acids to form 3-nitrotyrosine (3-NT) (Beckman et al., 1990), a stable marker of oxidative stress that is associated with CVD (Wattanapitayakul et al., 2000). Previous studies have shown that 3NT formation on isolated low density lipoproteins can facilitate the formation and uptake of foam cells into the blood vessel wall of RA patients (Griffiths et al., 2006). 3-NT may therefore represent a specific marker of interest when linking oxidative stress with CVD risk in RA.

Regular exercise is associated with a reduction in multiple cardiovascular risk factors (Shephard and Balady, 1999) as well as improved vascular function (Clarkson et al., 1999) and improved joint function (Häkkinen et al., 2004). Exercise training in RA has shown to improve patient health, with increased functional capacity and decreased disease activity reported (Metsios et al., 2008). In addition, our group has recently shown that individualised exercise improved CVD risk factors (Stavropoulos-Kalinoglou et al., 2012) and endothelial function (Metsios et al., 2013) in RA patients.

It is now well established that exercise can stimulate an acute rise in oxidative stress (Turner et al., 2011; Bloomer et al., 2005). There is evidence in healthy populations that over a period of training, the repetitive stimuli of exercise-induced RONS mediate increases in mitochondrial biogenesis (Ristow et al., 2009) and antioxidant enzyme protein expression (Khassaf et al., 2003) that result in lower levels of oxidative stress. In addition, exercise training is associated with reductions in systemic markers of inflammation, such as IL-8 (Trøseid et al., 2004) and C-reactive protein (Stewart et al., 2007), an acute phase protein implicated in atherosclerotic plaque development (Danesh and Pepys, 2009).

Early studies assessing oxidative stress in exercising RA patients demonstrated that isolated knee extension contractions can increase ROS production and subsequent oxidation of synovial biomolecules (Zhang et al, 1993; Edmonds et al, 2001; Blake et al, 1989). However, only one previous studies has assessed the impact of whole body exercise on oxidative stress in RA (Rall et al. 2000). Rall et al, 2000 reported no changes in urinary DNA oxidation following 12 weeks of resistance exercise. To our knowledge, no studies have explored the impact of whole body aerobic exercise on oxidative stress in RA, either following a single bout of exercise or repeated bouts of exercise. The aim of this study was to assess plasma markers of oxidative stress in response to an acute bout of exercise in untrained

RA patients. In addition, the impact of 3 months of moderate intensity aerobic exercise on plasma markers of oxidative stress and inflammation were examined.

3.3. Methods

3.3.1 Patients

RA patients were recruited from the rheumatology outpatient clinics of the Dudley Group NHS Foundation Trust following ethical approval from the Black Country ethical committee. All procedures took place at Dudley Group NHS Foundation Trust and the trial was registered with the ISRCTN register (ISRCTN50861407). All patients gave their informed written consent and experimental procedures were in accordance with the declaration of Helsinki. Inclusion criteria were: RA according to the American College of Rheumatology (ACR) 1987 revised criteria (Arnett, 1988), a sedentary lifestyle (not engaged in structured physical activity within the last 6 months) and stable disease activity (no changes in disease-modifying antirheumatic drugs (DMARDs) or intra-venous (IV) and intra-muscular (IM) steroids within the last 3 months). Exclusion criteria were: recent joint surgery or co-morbidity that would impact on the patient's ability to undertake regular exercise (ACSM, 2005). Upon entry into the training study, patients were randomised into exercise and control groups as described previously (Stavropoulos-Kalinoglou et al., 2012; Metsios et al., 2013).

3.3.2 Acute Exercise Study

3.3.2.1 Acute Exercise Protocol

All patients were required to visit the laboratory on two separate occasions. On the first visit, baseline assessments of heart rate, blood pressure, demographic and anthropometric data and disease activity were obtained. Patients then undertook an exercise tolerance test (ETT) to determine their cardiorespiratory fitness.

On a separate day, after an overnight fast, a blood sample was taken from the antecubital vein of the arm by an indwelling catheter. Patients then undertook a single bout of

exercise at a heart rate that elicited 70% maximum oxygen consumption (VO_{2MAX}) for 30-40 minutes (*Figure 3.1*). Heart rate monitors (*Polar S610i, Polar Electro Oy, Kempele, Finland*) were used to confirm adherence to the exercise intensity. Patients performed 3 circuits, with 1 minute of rest in-between each circuit. Each circuit consisted of 3-4 intervals, where patients were instructed to exercise for no more than 3-4 minutes per interval. Blood samples were taken before (*baseline*), immediately (*post+0*) and 30 minutes following exercise (*post+30*). All blood samples were centrifuged (3000 rpm at 8°C for 10 minutes) and plasma was stored at -80°C until further analyses. Due to complications with blood sample availability, data from the acute exercise study was available for only 12 of the 19 patients in the training study.

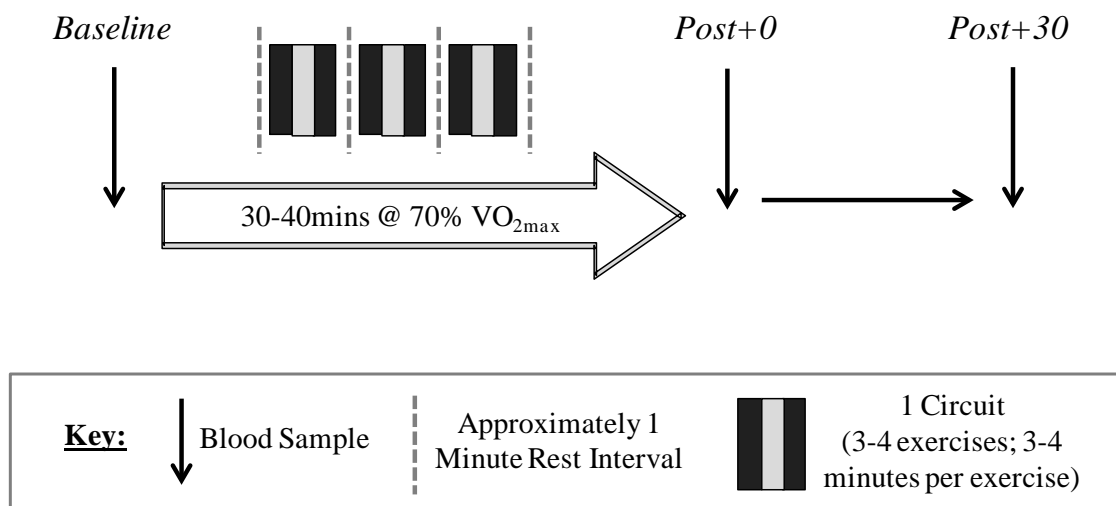


Figure 3.1: A schematic of the acute exercise assessment.

3.3.2.2. Exercise tolerance test

Cardiorespiratory fitness was assessed by determining the VO_{2MAX} of patients. All patients were assessed on a treadmill (HP Cosmos, sports & medical gmbh, Nussdorf-Traunstein, Germany). A breath-by-breath system (Metalyzer 3B, CORTEX Biophysik

GmbH, Leipzig, Germany) was used for continuous measurement of oxygen uptake (VO_2), lung ventilation and cardiorespiratory parameters. In addition, heart rate was monitored during the test. After familiarisation, an incremental test (4-7km/h), with increasing inclination of the treadmill by 1% every 30 seconds was used until patients reached volitional exhaustion. $\text{VO}_{2\text{MAX}}$ was corrected for the weight of the patients and expressed in $\text{ml.kg}^{-1}\text{min}^{-1}$.

3.3.2.3 Analytical Procedures

Blood Assessments

Haemoglobin (g/dL) and haematocrit (%) were assessed to calculate plasma volume changes as a result of exercise (Kargotich et al., 1997) (*Coulter Analyser, Beckman-Coulter, High Wycombe, UK*). All reagents purchased from Sigma unless stated (*Sigma Aldrich, Dorset, UK*).

Protein Carbonylation (PC)

PC was assessed by ELISA (Buss et al., 1997; Carty et al., 2000) in order to quantify the degree of protein oxidation in plasma samples. Samples were diluted in coating buffer (50mM sodium carbonate, pH=9.2) to a concentration of 0.05mg/ml. Samples and standards (50 μ l) were added in triplicate to a 96 well NUNC maxisorb microtitre plate for 1 hour at room temperature. Bound protein was then incubated in the dark for 1 hour at room temperature with 2, 4-dinitrophenylhydrazine (DNPH) (1mM, in 2M HCl). All wells were then blocked (200 μ l) with TBS Tween (0.1%) overnight at 4°C. Wells were incubated with monoclonal mouse anti-DNP antibody (50 μ l, 1:1000) for 2 hours at room temperature, followed by peroxidase conjugated rat anti-mouse IgE conjugated HRP (50 μ l, 1:5000, *AbD Serotec, Oxford, UK*) for 1 hour at room temperature. All steps were followed by three

washes to the 96 well plate using TBS Tween (0.05%). Substrate (0.5M Citrate phosphate buffer (10mls, pH=5), hydrogen peroxide (8µl) and Ortho-Phenylenediamine tablet (2mg); 50µl) was added to each well and the reaction stopped after 20-25 minutes with 2M sulphuric acid (50µl). Each well was measured for absorbance at 490nm (Multiscan MS, Labsystems) and quantified using absorbance values of PC standards made in our lab (1.28-5.20 nmols/mg of protein). This method is detailed in section 2.3.8. Protein concentration was determined using the bicinchoninic assay method (Smith et al., 1985), described in section 2.3.8.

Lipid Hydroperoxides (LOOH)

LOOH concentrations were assessed using a spectrophotometric assay (El-Saadani et al., 1989). Samples and a blank standard (10µl) were added to a 96 well microtitre plate in triplicate. Reagent mix (100µl, 0.2M Potassium phosphate (pH=6.2), 0.12M potassium iodide, 0.15mM sodium azide, Triton X (2g/l), alkylbenzyltrimethylammonium (0.1g/l), 10µM ammonium molybdate and HPLC grade water (to make total volume 100mls)) was added for 30 minutes at room temperature, away from light on a plate shaker. The plate was read at 340nm (Multiscan MS, Labsystems), concentration of lipid peroxides (µmol/l) determined using the Beer-Lambert Law (extinction co-efficient $\epsilon_{340} = 24600 \text{ M}^{-1}\text{cm}^{-1}$) and adjusted for changes in plasma volume (Kargotich et al., 1997).

Total Antioxidant Capacity (TAC)

TAC was assessed using the Ferric Reducing Ability of Plasma (FRAP) assay (Benzie and Strain, 1996). Plasma samples (10 µl per well) and standards (ascorbic acid, 0-1000 µM; 10 µl) were added in triplicate to a flat bottomed 96 well plate. FRAP reagent (300mM sodium acetate (pH=3.6), 160mM 2, 4, 6- tripyridyltriazine and 20mM ferric chloride (FeCl_3); 300µl) was added to each well and left to incubate for 8 minutes at room temperature, then

absorbances read at 650nm. TAC values were obtained using absorbance values of known ascorbic acid concentrations (0-1000 μ M), expressed as μ M of antioxidant power relative to ascorbic acid (McAnulty et al., 2005) and adjusted for changes in plasma volume (Kargotich et al., 1997).

Total Nitric Oxide Metabolites (NO_x)

The Griess assay was used to quantify NO_x in plasma samples (Miranda et al., 2001; Moshage et al., 1995). Samples (100 μ l) were diluted with HPLC grade water (375 μ l) and zinc sulphate (25 μ l, 300mg/ml) to give a concentration of approximately 15mg/ml. Samples were vortexed and then centrifuged at 10,000g for 20 minutes (10°C). Supernatants and standards (100 μ l) were added to a 96 well microtitre plate, and 100 μ l vanadium (III) chloride (8mg/ml) then added. Thorough mixing was ensured to fully reduce plasma nitrate to nitrite. Sulphanilamide (50 μ l, 2%) and N-(1-naphthyl) ethylenediamine dihydrochloride (50 μ l, 0.1%) were then rapidly added to each well. Plates were incubated for 30 minutes at 37°C, absorbances read at 540nm and compared with values of known nitrite concentrations (0-100 μ M). Values were adjusted for changes in plasma volume (Kargotich et al., 1997).

3.3.2.4 Assessments

Demographic & anthropometric assessments

Demographic data was collected by questionnaire. Height and weight were measured to the nearest 0.5cm (Seca 214 Road Rod) and 0.5kg (B (Tanita C-418 MA Segmental Body Composition Analyser, Tanita Corporation, Tokyo, Japan) respectively, and body mass index (BMI) was subsequently calculated.

Pulse Rate & Blood Pressure

Pulse rate and Blood pressure (BP) was assessed following at least 5 minutes of rest, on the right arm with the patient in a seated position. Reported values represent the mean of three readings taken at 5 minute intervals.

RA assessments

Clinical disease activity (DAS28) was assessed in accordance with a validated 28-joint count system (Prevoo, 1995).

3.3.3. Exercise Training Study

3.3.3.1. Baseline Assessments

Baseline demographic, anthropometric and disease related measures, as well as cardiorespiratory fitness were assessed as described in the acute exercise study. As before, a fasted blood sample was taken from the antecubital vein of the arm. Serum and plasma (after centrifuging at 3000 rpm at 8°C for 10 minutes) were stored at -80°C until further analysis. Triglycerides, cholesterol and high density lipoprotein cholesterol (HDL-C) were analysed immediately using a Vitros 5.1 FS Chemistry System.

3.3.3.2 Exercise Training Protocol

The exercise training protocol is described in detail elsewhere (Stavropoulos-Kalinoglou et al., 2012; Metsios et al., 2013). As described above, 12 patients from the acute exercise study continued into the exercise training study, alongside 7 additional RA patients. Briefly, exercise training commenced within two weeks of the ETT. Patients in the control group were given advice on the benefits of exercise throughout the same period. Whenever possible, patients in both groups and their managing consultants were asked to avoid changes

to DMARDs or IV and IM steroid use. Patients in the exercise group undertook three sessions per week, two semi-supervised by a qualified exercise physiologist and one session performed in their own time. In each session, patients worked at a heart rate that elicited 70% $\text{VO}_{2\text{MAX}}$ (Figure 3.2). Heart rate monitors (*Polar S610i, Polar Electro Oy, Kempele, Finland*) were attached to the sternum of patients during each session to confirm adherence to the prescribed exercise intensity. Depending on patient preference and the physiologists' assessment of the perceived ability of the patient, the exercise prescription used a treadmill, cycle, hand or rowing ergometer. Patients performed 3 circuits per session with 1 minute of rest in-between each circuit. Each circuit consisted of 3-4 intervals, where patients were instructed to exercise for no more than 3-4 minutes per interval (total exercise 30-40 minutes). Following the 3 month period, all baseline assessments were repeated in both groups (Figure 3.2).

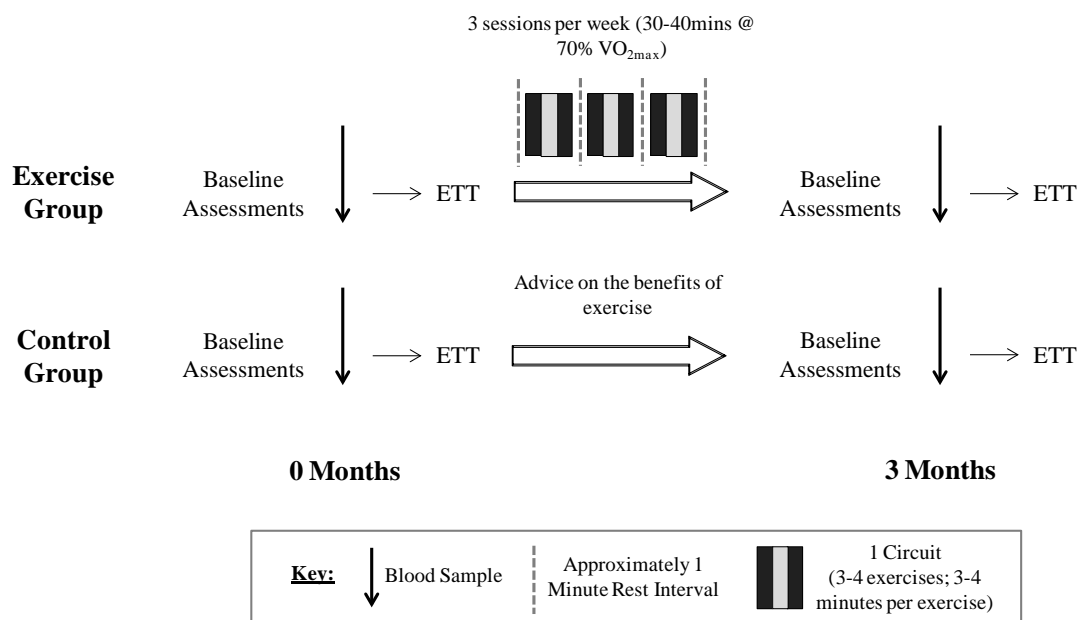


Figure 3.2: A schematic of the 3 month protocol for exercise and control groups.

3.3.3.3 Analytical Procedures

PC, LOOH, TAC and NO_x were assessed as in the acute exercise study. All reagents purchased from Sigma unless stated (*Sigma Aldrich, Dorset, UK*).

Free & Protein Bound 3-Nitrotyrosine

Free and protein-bound plasma 3-nitrotyrosine (3-NT) was quantified using High Performance Liquid Chromatography with electrochemical detection (HPLC-EC), based on the method described by Maruyama et al. (1996). Mobile phase (Citric Acid (50mM, pH=3.1), Phosphoric Acid (50mM), Octane Sulphonic Acid (100 mg/l), Ethylenediaminetetraacetic acid (EDTA) (40 mg/l) and methanol (5%)) was made fresh daily and set at 1.0 ml/min flow rate (applied voltage = 990mV). Total free and bound 3-nitrotyrosine was determined by diluting samples to 1mg/ml in Calcium Chloride (10mM) and hydrolysed with Pronase (20µg/L). Samples were spiked with known concentrations of synthetic 3-NT (10µM) and loaded (20µl) onto a C18 column (Phenomenex, Luna 3µM, C18 (2) 100A, 150x4.60mm). In addition, daily quality controls were run (3-NT, 10µM) and used for the calibration of the HPLC, alongside weekly standard curves (0-20 µM) for subsequent quantification of plasma 3-NT (see *figure 3.3*). Levels of 3-nitrotyrosine were calculated by subtracting the peak area (mV*min) of the synthetic 3-nitrotyrosine standard (10µM) from the peak area of the spiked sample (plasma 3-NT + 10 µM standard) (example chromatograms, *figure 3.4*). Samples were analysed in duplicate.

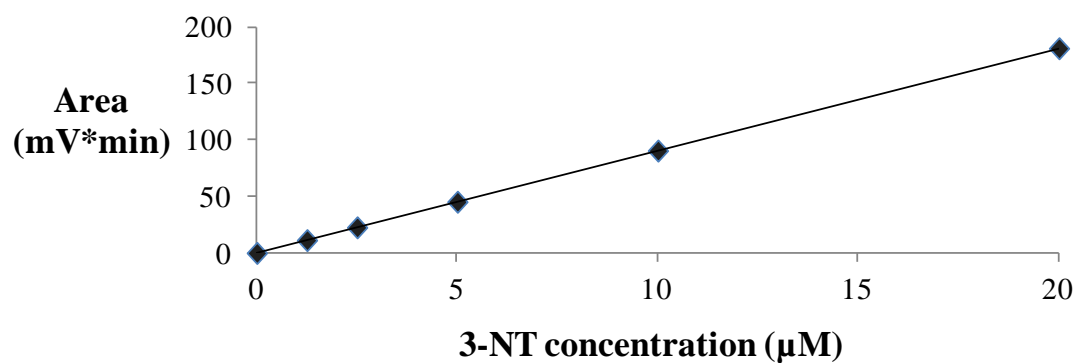


Figure 3.3 3-Nitrotyrosine standard curve (0 – 20 μM)

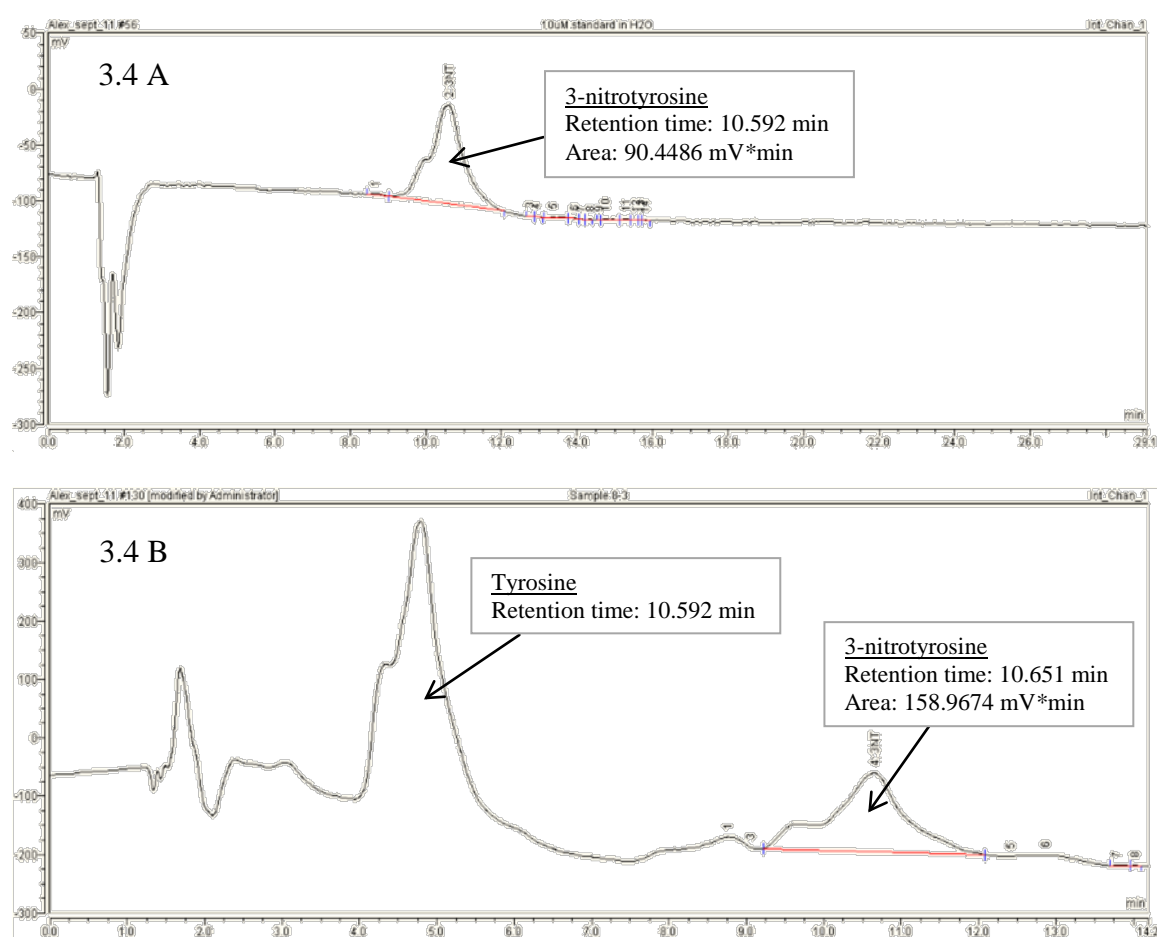


Figure 3.4 Example chromatograms from HPLC-EC analysis of synthetic and plasma 3-NT. The area (mV*min) from the synthetic 10 μM 3-NT standard (3.4 A) was subtracted from the combined area of the plasma 3-NT and 10 μM spike (3.4 B).

Catalase Enzyme Activity

Peroxidatic activity of Catalase was measured in plasma (diluted 1:1 with sample buffer [25mM potassium phosphate, pH = 7.5, 1mM EDTA and 0.1%BSA]), using a commercially available assay kit according to manufacturer instructions (*Cayman Chemical*). In brief, samples were incubated with methanol, in presence of hydrogen peroxide, and formaldehyde formation measured as an indicator of enzyme activity. After addition of a chromogen (*purpald*), cell absorbance's were measured spectrophotometrically at 540nm and compared with absorbances of standards of known formaldehyde concentration (0-75 μ M).

C-Reactive Protein

Serum concentrations of CRP (mg/dL) were obtained using the Vitros® 5.1 FS Chemistry system by a clinical biochemist at Dudley hospital. In brief, samples were added to a slide alongside phosphorylcholine bound polystyrene polymer beads, which can bind CRP. A monoclonal anti-CRP antibody and lueco detection dye were then added to the slide and the reflection density of the dye and CRP-antibody complex was proportional to amount of CRP in the sample.

Interleukin-8 (IL-8)

Plasma concentrations of IL-8 were determined using a commercially available ELISA kit, according to manufacturer instructions (*Sanquin, PeliKine*). Briefly, samples (diluted 1:5 with dilution buffer [Sanquin]) were incubated with a monoclonal antibody derived against IL-8 and then substrate solution (12ml substrate buffer, 0.11M acetate buffer, pH=5.5; 200 μ l 3,5,3',5'-tetramethylbenzidine, 6mg/ml in dimethyl sulfoxide; 12 μ l hydrogen peroxide, 3%) added and absorbances read at 450nm. Values were obtained from a linear standard curve of known IL-8 concentrations (0-240pg/ml).

3.3.3.4. Statistical Analysis

Statistical analyses were performed using SPSS (PASW Statistics, release 18.0, SPSS Inc., Chicago, IL, USA). Measurements taken in the acute exercise study were analysed by 3 time (pre, post+0 and post+30) repeated measures analyses of variance (ANOVA). In the training study, potential baseline differences in demographic, fitness, disease activity, inflammation or oxidative stress variables between the exercise and control group were assessed using independent t-tests. Changes as a result of exercise training were assessed by 2 group (exercise, control) by 2 time (baseline, 3 months) repeated-measures ANOVA. The Kolmogorov Smirnov test was used to test for normally distributed data at all timepoints. Data was log transformed where appropriate (Log10). Statistical significance was accepted at the $p < .05$ level.

3.4. Results

3.4.1. Acute Response to Exercise

3.4.1.1. Oxidative stress response to exercise

Table 3.1 describes the characteristics of the patients in the acute exercise study. Changes in response to exercise of NO_x, TAC, LOOH and PC (before [baseline], immediately [post+0] and 30 minutes following exercise [post+30]) are shown in Figure 3.3 (A-D). Exercise caused a significant increase in NO_x ($p=.040$) and PC ($p=.033$). Post hoc analyses revealed that PC was significantly different from pre-exercise values post+30 ($p=.002$). No statistical differences were observed in TAC or LOOH in response to exercise ($p's>.05$).

Table 3.1: Mean (SD) patient characteristics in the acute exercise study.

Patient Characteristics	(N=12)
Age (yrs)	56 (11)
Weight (kg)	78.46 (14.23)
BMI (kg.m ⁻²)	28.61 (4.97)
VO ₂ MAX (ml.kg ⁻¹ min ⁻¹)	25.31 (5.31)
Resting Heart Rate (bpm)	74.3 (10.95)
Mean Arterial Pressure (mmHg)	97.83 (6.93)
Disease Activity Scores (DAS28)	2.98 (1.17)

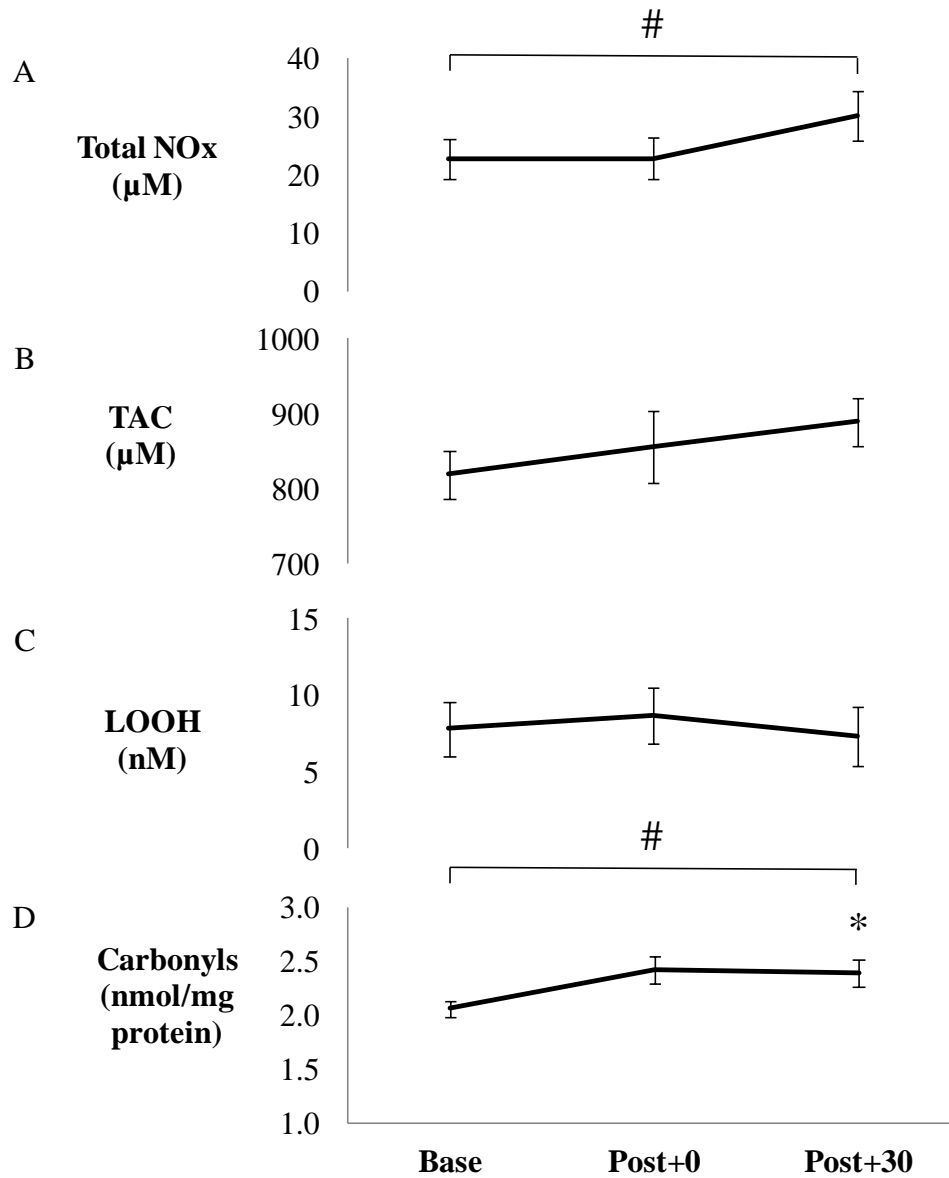


Figure 3.5: Acute oxidative stress response to exercise in RA. Nitric oxide metabolites (A), Total antioxidant capacity (B), Lipid hydroperoxides (C) & Protein carbonylation (D). Data are means \pm standard error, * $p < 0.05$ significantly different from baseline. # $p < 0.05$ indicates a main effect of time.

3.4.2. Exercise Training Study

3.4.2.1. Baseline measures

Characteristics of the patients in the exercise and control group at baseline are reported in Table 3.2. There were no group differences in any of the variables reported in Table 3.2 (Independent t-test).

Table 3.2: Mean (SD) patient characteristics in the exercise training study at baseline.

Patient Characteristics	Control (N=7)	Exercise (N=12)
Age (yrs)	53 (12)	57 (9)
Weight (kg)	78.5 (13.04)	79.03 (15.28)
BMI (kg.m ⁻²)	28.30 (4.16)	29.08 (6.63)
Disease Duration (yrs)	7 (4)	7 (7)
VO _{2MAX} (ml.kg ⁻¹ min ⁻¹)	25.01 (4.58)	24.45 (4.98)
Resting Heart Rate (bpm)	79.14 (10.39)	70.33 (10.47)
Mean Arterial Pressure (mmHg)	92.71 (9.72)	96.75 (5.81)

3.4.2.2. Markers of disease activity, aerobic fitness, nitric oxide metabolism and cardiovascular risk factors in response to exercise training

The effect of exercise on DAS28, VO_{2MAX} and cardiovascular risk factors are shown in Table 3.3. DAS28 and VO_{2MAX} were unchanged in the control group, whereas DAS28 decreased (interaction effect, $p=.004$) and VO_{2MAX} increased (interaction effect, $p=.001$) in the exercise group. No changes were observed in either group in weight, BMI, or lipid levels ($p's>.05$).

Table 3.3: Mean (SD) disease activity, aerobic fitness, nitric oxide metabolites & cardiovascular risk factors values at baseline and following 3 months of training.

	Control		Exercise	
	Baseline	3 Months	Baseline	3 Months
Disease Activity (DAS28)	2.43 (0.84)	2.79 (0.68)	3.47 (1.17)	2.88 (0.76)*
VO ₂ MAX (ml.kg ⁻¹ .min ⁻¹)	25.01 (4.58)	22.77 (3.45)	24.45 (4.98)	27.10 (4.51)*
Weight (kg)	78.5 (13.04)	79.11 (13.10)	79.03 (15.28)	78.01 (14.69)
BMI (kg.m ⁻²)	28.30 (4.16)	28.53 (4.25)	29.08 (5.63)	28.70 (5.40)
NOx (μM)	25.97 (12.43)	29.71 (15.04)	22.71 (12.18)	25.29 (12.96)
Cholesterol (mg/dL)	4.73 (1.08)	4.76 (1.06)	4.97 (1.15)	4.88 (1.23)
Triglycerides (mg/dL)	1.33 (0.63)	1.27 (0.69)	1.41 (0.50)	1.38 (0.95)
HDL-C (mmol/L)	1.59 (0.36)	1.61 (0.23)	1.34 (0.47)	1.34 (0.40)

*p < .05 significantly different from baseline.

3.4.2.3. Markers of oxidative stress in response to exercise training

The effects of exercise on markers of oxidative stress are shown in Table 3.4. No changes were observed in catalase enzyme activity, logLOOH, PC or logTAC in either the control or exercise groups (time and interaction effects, p's>.05). A non-significant increase in TAC was observed in the exercise group only, but this did not reach statistical significance (interaction effect, p=.113). However, a significant group x time interaction effect was found for levels of free and bound 3-nitrotyrosine (p=.027). Post hoc analyses revealed that 3-nitrotyrosine was unchanged in the control group, whereas it was decreased in the exercise group (Figure 3.4).

Table 3.4: Mean (SD) markers of oxidative stress at baseline and in response to 3 months training in RA patients.

	Control		Exercise	
	Baseline	3 Months	Baseline	3 Months
Protein carbonyls (nmol/mg protein)	1.79 (0.28)	1.82 (0.15)	1.78 (0.13)	1.76 (0.25)
Lipid Hydroperoxides (nM)	8.69 (5.56) IQ = 10.35	9.01 (6.39) IQ = 11.83	7.04 (6.63) IQ = 6.47	5.07 (5.12) IQ = 4.74
Total free and bound 3-Nitrotyrosine (μM)	0.947 (0.75)	1.626 (1.27)	2.175 (1.78)	1.101 (0.93)*
Catalase activity (nmol/min/ml)	46.33 (13.17)	50.10 (12.76)	44.21 (14.76)	46.30 (6.94)
Total antioxidant capacity (μM: equivalent to ascorbic acid)	763.81 (67.81) IQ = 64.44	763.81 (160.67) IQ = 173.33	812.12 (108.99) IQ = 202.22	926.06 (242.38) IQ = 253.33

*p < .05 (repeated measures ANOVA) is a decrease compared with baseline values.

Interquartile range (IQ) is reported for log transformed data.

3.4.2.4. Markers of inflammation in response to exercise training

The effects of exercise on markers of inflammation are shown in Table 3.5. No changes were observed in IL-8 or logCRP in either the exercise and control groups.

Table 3.5: Mean (SD) markers of inflammation in response to 3 months training in RA patients.

	Control		Exercise	
	Baseline	3 Months	Baseline	3 Months
Interleukin-8 (pg/ml)	7.12 (± 2.13)	7.60 (± 2.77)	6.52 (± 1.86)	6.16 (± 1.06)
CRP (mg/L)	3.43 (± 0.43) IQ = 0.5	5.43 (± 1.09) IQ = 3	8.17 (± 1.75) IQ = 9	3.92 (± 0.53) IQ = 2

Interquartile range (IQ) is reported for log transformed data.

3.5. Discussion

This is the first study to examine the effects of both an acute bout and repeated whole body aerobic exercise sessions on markers of oxidative stress in untrained patients with RA. Plasma PC and NOx increased in response to a single bout of moderate intensity exercise (Figure 3.3). Following 3 months of exercise training at the same intensity as the acute bout, we observed a decrease in plasma 3-NT. PC, LOOH, TAC or CAT remained unchanged post-training when compared to pre-training values (Table 3.4). In line with previous studies, disease activity was reduced and aerobic fitness increased in the exercise training group only (Table 3.3).

Acute moderate intensity exercise ($\approx 60\text{-}70\%$ $\text{VO}_{2\text{MAX}}$) has previously been shown to cause an increase in PC (Bloomer et al., 2007), LOOH (Turner et al., 2011) and TAC (Berzosa et al., 2011) in young healthy males. The current study reports an increase in PC, however no significant changes in LOOH or TAC following a single exercise bout in RA patients. Protein carbonylation represents a global, rather than specific, oxidative modification to proteins, which can be indicative of both targeted proteolytic degradation and post translational modification (Dalle-Donne et al., 2006). Interestingly, the degree (\pm nmol/mg protein) of change in PC relative to baseline in the current study was comparable to that reported in a previous study of similar intensity and duration (70% $\text{VO}_{2\text{MAX}}$, 30 minutes) in young, healthy men and women (Bloomer et al., 2007). Antioxidant capacity may decrease during exercise as antioxidants quench radicals that are released, but studies generally report increases in antioxidant capacity in healthy subjects (Turner et al., 2011), as soluble antioxidants are mobilised from vessel walls (Gleeson, M., Robertson, J.D., Maughan, 1987). In RA, there is evidence that TAC is lower in comparison to age-matched controls at rest (Sarban et al., 2005). In the current study, TAC increased thirty minutes following exercise, relative to baseline (+11.4%), but this increase did not reach statistical significance.

Nevertheless, these data collectively indicate that a single bout of moderate intensity exercise caused a transient change in markers of oxidative stress comparable with those observed in healthy populations.

Following three months of regular moderate intensity exercise, a decrease in total free and bound plasma 3-NT was observed. Given that no alterations were observed in NO_x, CRP or IL-8 following 3 months of exercise training (Table 3.5), it is unlikely that this decrease was driven by altered NO production, which is sometimes seen following exercise training where systemic inflammation is perturbed (Gielen et al., 2003). Similar decreases in LOOH, PC, TAC and CAT were not observed following 3 months of training, however, it must be noted that these levels at baseline were low in comparison to previous studies in RA (Seven et al., 2008) and studies in our laboratory in healthy cohorts (Turner et al., 2011). While this may be a reason why decreases in global markers of oxidative stress were not observed, the decrease in 3NT may represent a specific and functional post-exercise protein modification. Given that previous evidence has highlighted an important role for 3NT in linking oxidative stress with vascular pathology (Griffiths et al., 2006), this finding warrants further study.

There is limited data available assessing the significance of acute increases in oxidative stress in response to exercise in clinical populations. Whilst emerging evidence in healthy populations strongly suggests that repeated exercise-induced increases in oxidative stress are adaptive (Ristow et al., 2009), a theoretical ‘threshold’ may exist, whereby exercise-induced RONS production could compromise redox status, and be detrimental to health (Goto et al., 2007). Indeed, previous studies in patients with heart disease have reported an exaggerated oxidative stress response following an exhaustive bout of exercise when compared to age-matched controls (Nishiyama et al., 1998; Avogaro et al., 1986). In the context of RA, RONS are thought to play a significant role in progressive joint degradation and risk for CVD (Mapp et al., 1995; Winrow et al., 1993). This is the first study

to demonstrate that a period of moderate intensity training does not exacerbate basal levels of oxidative stress in RA patients with stable disease activity. In conjunction with data from the acute exercise study, transient increases in oxidative stress following moderate intensity exercise may be beneficial, providing further support for the use of moderate intensity exercise in RA.

This study is not without limitations. Firstly, an age-matched non-RA control group was not used in the acute exercise study to compare the differences in the oxidative stress response to exercise between non-RA and RA patients. However, the results presented clearly show that exercise did not stimulate an exaggerated oxidative stress response to exercise in RA, when compared to other studies assessing similar outcome measures in healthy populations (Turner et al., 2011; Bloomer et al., 2007). In the exercise training study, the sample size used was small and the disease activity of the RA patients was relatively low. Although statistically significant baseline differences in markers such as 3-NT and CRP were not observed between exercise and control groups, this may be attributable to low statistical power. However, in the context of training-induced reductions in plasma 3-NT, it must be noted that 9 of the 12 subjects in the exercise group demonstrated a marked reduction in total and bound 3-NT, supporting a robust finding. Nevertheless, with no other data available, to our knowledge, exploring oxidative stress in response to whole body aerobic exercise in RA, these preliminary results are perhaps applicable only to patients with stable disease activity. Changes to patient medication were not prevented in this study, in either the exercise or control group, however no patients reported any changes throughout the 3 month period.

The results of the current study provide data showing that oxidative stress is increased in response to a single bout of moderate intensity exercise in RA patients. Three months of moderate intensity exercise training increased aerobic fitness, decreased disease activity and decreased plasma protein nitration. All other markers of oxidative stress and inflammation

were unchanged following exercise training, indicating that exercise did not cause prolonged or significant oxidative stress. These data demonstrate that transient exercise-induced increases in oxidative stress are not harmful in RA patients with stable disease activity, but in concurrence with studies in healthy populations, may be adaptive. Further research is required to clarify this in RA.

3.6. Reference list for chapter three

ACSM (2005) “Guidelines for exercise testing and prescription.” In **7th ed. Philadelphia, Pa: Lippincott Williams & Wilkins**. pp. 286 –299

Arnett, F.. (1988) The American Rheumatism association 1987 revised criteria for the classification of Rheumatoid Arthritis. **Arthritis and rheumatism**, 31: 315–324

Avogaro, P., Bon, G.B., Cazzolato, G., et al. (1986) Plasma Malondialdehyde in Coronary Patients and in “ Normal ” People at Rest and After Exercise. **Cardiovascular Medicine and Surgery**, 13: 291–296

Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. **Journal of Clinical Investigation**, 52 (3): 741–744

Beckman, J.S., Beckman, T.W., Chen, J., et al. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. **Proc Natl Acad Sci U S A**, 87 (4): 1620–1624

Benzie, I.F. and Strain, J.J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. **Analytical Biochemistry**, 239 (1): 70–76

Berzosa, C., Cebrian, I., Fuentes-Broto, L., et al. (2011) Acute exercise increases plasma total antioxidant status and antioxidant enzyme activities in untrained men. **J Biomed Biotechnol**, pp. 1–7

Biemond, P., Swakk, A.J., Penders, J.M., et al. (1986) Superoxide production by polymorphonuclear leucocytes in rheumatoid arthritis and osteoarthritis: in vivo inhibition by the antirheumatic drug piroxicam due to interference with the activation of the NADPH-oxidase. **Annals of the rheumatic diseases**, 45 (3): 249–55

Blake, D.R., Unsworth, J., Outhwaite, J.M, et al. (1989) Hypoxic-reperfusion injury in the inflamed human joint. **The Lancet**, 333 (8633): 289-293

Bloomer, R.J., Davis, P.G., Consitt, L.A., et al. (2007) Plasma protein carbonyl response to increasing exercise duration in aerobically trained men and women. **International Journal of Sports Medicine**, 28 (1): 21–25

Bloomer, R.J., Goldfarb, A.H., Wideman, L., et al. (2005) Effects of acute aerobic and anaerobic exercise on blood markers of oxidative stress. **Journal of strength and conditioning research / National Strength & Conditioning Association**, 19 (2): 276–85

- Buss, H., Chan, T.P., Sluis, K.B., et al. (1997) Protein carbonyl measurement by a sensitive ELISA method. **Free Radical Biology and Medicine**, 23 (3): 361–366
- Carty, J.L., Bevan, R., Waller, H., et al. (2000) The effects of vitamin C supplementation on protein oxidation in healthy volunteers. **Biochem Biophys Res Commun**, 273 (2): 729–735
- Clarkson, P., Montgomery, H.E., Mullen, M.J., et al. (1999) Exercise training enhances endothelial function in young men. **Journal of the American College of Cardiology**, 33 (5): 1379–85
- Dalle-Donne, I., Aldini, G., Carini, M., et al. (2006) Protein carbonylation, cellular dysfunction, and disease progression. **J Cell Mol Med**, 10 (2): 389–406
- Danesh, J. and Pepys, M.B. (2009) C-reactive protein and coronary disease: is there a causal link? **Circulation**, 120 (21): 2036–9
- Edmonds, S., Turnbull, C. and Blake, D. (2001) Effect of intensive exercise on patients with active RA. **Ann Rheum Dis**, 60: 429
- El-Saadani, M., Esterbauer, H., El-Sayed, M., et al. (1989) A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. **Journal of Lipid Research**, 30 (4): 627–630
- Farrell, A.J., Blake, D.R., Palmer, R.M., et al. (1992) Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. **Annals of the rheumatic diseases**, 51 (11): 1219–22
- Fu, Y., Luo, N. and Lopes-Virella, M.F. (2002) Upregulation of interleukin-8 expression by prostaglandin D2 metabolite 15-deoxy-delta12, 14 prostaglandin J2 (15d-PGJ2) in human THP-1 macrophages. **Atherosclerosis**, 160 (1): 11–20
- Gielen, S., Adams, V., Möbius-Winkler, S., et al. (2003) Anti-inflammatory effects of exercise training in the skeletal muscle of patients with chronic heart failure. **Journal of the American College of Cardiology**, 42 (5): 861–868
- Gleeson, M., Robertson, J.D., Maughan, R.J. (1987) Influence of exercise on ascorbic acid status in man. **Clin Sci (Lond)**, 73 (5): 501–505
- Goto, S., Naito, H., Kaneko, T., et al. (2007) Hormetic effects of regular exercise in aging: correlation with oxidative stress. **Appl Physiol Nutr Metab**, 32 (5): 948–953
- Griffiths, H.R., Aldred, S., Dale, C., et al. (2006) Homocysteine from endothelial cells promotes LDL nitration and scavenger receptor uptake. **Free Radical Biology and Medicine**, 40 (3): 488–500

- Häkkinen, A., Sokka, T., Kautiainen, H., et al. (2004) Sustained maintenance of exercise induced muscle strength gains and normal bone mineral density in patients with early rheumatoid arthritis: a 5 year follow up. **Annals of the rheumatic diseases**, 63 (8): 910–6
- John, H., Kitas, G., Toms, T., et al. (2009) Cardiovascular co-morbidity in early rheumatoid arthritis. **Best practice & research. Clinical rheumatology**, 23 (1): 71–82
- Kargotich, S., Goodman, C., Keast, D., et al. (1997) The influence of exercise-induced plasma volume changes on the interpretation of biochemical parameters used for monitoring exercise, training and sport. **Sports medicine**, 7 (3): 185–191
- Kaur, H. and Halliwell, B. (1994) Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. **FEBS Lett**, 350 (1): 9–12
- Khassaf, M., McArdle, A., Esanu, C., et al. (2003) Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. **Journal of Physiology**, 549 (2): 645–652
- Kitas, G.D. and Gabriel, S.E. (2011) Cardiovascular disease in rheumatoid arthritis: state of the art and future perspectives. **Annals of the rheumatic diseases**, 70 (1): 8–14
- Lee, D.M. and Weinblatt, M.E. (2001) Rheumatoid arthritis. **Lancet**, 358 (9285): 903–911
- Mapp, P.I., Grootveld, M.C. and Blake, D.R. (1995) Hypoxia, oxidative stress and rheumatoid arthritis. **Br Med Bull**, 51 (2): 419–436
- Maruyama, W., Hashizume, Y., Matsubara, K., and Naoi, M. (1996). Identification of 3-nitro-L-tyrosine, a product of nitric oxide and superoxide, as an indicator of oxidative stress in the human brain. **J. Chromatogr. B Biomed.** 676: 153-158.
- McAnulty, S.R., McAnulty, L.S., Morrow, J.D., et al. (2005) Effect of daily fruit ingestion on angiotensin converting enzyme activity, blood pressure, and oxidative stress in chronic smokers. **Free Radic Res**, 39 (11): 1241–1248
- Metsios, G.S., Stavropoulos-Kalinoglou, A., Veldhuijzen van Zanten, J.J., et al. (2013) Individualised exercise improves endothelial function in patients with rheumatoid arthritis. **Annals of the rheumatic diseases**, pp. 1–4
- Metsios, G.S., Stavropoulos-Kalinoglou, A., Veldhuijzen van Zanten, J.J.C.S., et al. (2008) Rheumatoid arthritis, cardiovascular disease and physical exercise: a systematic review. **Rheumatology (Oxford)**, 47 (3): 239–248
- Miranda, K.M., Espey, M.G. and Wink, D.A. (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. **Nitric Oxide**, 5 (1): 62–71

- Miyata, T., Ishiguro, N., Yasuda, Y., et al. (1998) Increased Pentosidine, an Advanced Glycation End Product, in Plasma and Synovial Fluid from Patients with Rheumatoid Arthritis and Its Relation with Inflammatory Markers. **Biochemical and Biophysical Research Communications**, 224 (1): 45–49
- Moshage, H., Kok, B., Huizenga, J.R., et al. (1995) Nitrite and nitrate determinations in plasma: a critical evaluation. **Clin Chem**, 41 (6 Pt 1): 892–896
- Nishiyama, Y., Ikeda, H., Haramaki, N., et al. (1998) Oxidative stress is related to exercise intolerance in patients with heart failure. **American Heart Journal**, 135: 115–120
- Prevoo, M.L.L. (1995) Modified disease activity scores that include twenty-eight-joint counts. **Arthritis & Rheumatism**, 38 (1): 44–48
- Rall LC, Roubenoff R, Meydani SN, et al. (2000) Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) as a marker of oxidative stress in rheumatoid arthritis and aging: effect of progressive resistance training. *J Nutr Biochem* 11:581–584.
- Ristow, M., Birringer, M., Kiehnopf, M., et al. (2009) Antioxidants prevent health-promoting effects. **PNAS**, 106 (21): 8865–8670
- Sarban, S., Kocyigit, A., Yazar, M., et al. (2005) Plasma total antioxidant capacity, lipid peroxidation, and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. **Clinical biochemistry**, 38 (11): 981–6
- Seven, A., Guzel, S., Aslan, M., et al. (2008) Lipid, protein, DNA oxidation and antioxidant status in rheumatoid arthritis. **Clinical Biochemistry**, 41 (7-8): 538–543
- Shephard, R.J. and Balady, G.J. (1999) Exercise as cardiovascular therapy. **Circulation**, 99 (7): 963–972
- Smith, P.K., Krohn, R.I., Hermanson, G.T., et al. (1985) Measurement of protein using bicinchoninic acid. **Analytical Biochemistry**, 150 (1): 76–85
- Stavropoulos-Kalinoglou, A., Metsios, G.S., Veldhuijzen van Zanten, J.J.C., et al. (2012) Individualised aerobic and resistance exercise training improves cardiorespiratory fitness and reduces cardiovascular risk in patients with rheumatoid arthritis. **Annals of the rheumatic diseases**, pp. 1–7
- Stewart, L.K., Flynn, M.G., Campbell, W.W., et al. (2007) The influence of exercise training on inflammatory cytokines and C-reactive protein. **Medicine and science in sports and exercise**, 39 (10): 1714–9
- Trøseid, M., Lappegård, K.T., Claudi, T., et al. (2004) Exercise reduces plasma levels of the chemokines MCP-1 and IL-8 in subjects with the metabolic syndrome. **European heart journal**, 25 (4): 349–55

Turner, J.E., Bosch, J.A., Drayson, M.T., et al. (2011) Assessment of oxidative stress in lymphocytes with exercise. **Journal of applied physiology (Bethesda, Md. : 1985)**, 111 (1): 206–11

Vasanthi, P., Nalini, G. and Rajasekhar, G. (2009) Status of oxidative stress in rheumatoid arthritis. **Int J Rheum Dis**, 12 (1): 29–33

Wadley, A.J., Veldhuijzen van Zanten, J.J.C.S. and Aldred, S. (2012) The interactions of oxidative stress and inflammation with vascular dysfunction in ageing : the vascular health triad. **AGE**, 35 (3): 705–718

Wattanapitayakul, S.K., Weinstein, D.M., Holycross, B.J., et al. (2000) Endothelial dysfunction and peroxynitrite formation are early events in angiotensin-induced cardiovascular disorders. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology**, 14 (2): 271–8

Wink, D.A., Hanbauer, I., Grisham, M.B., et al. (1996) Chemical biology of nitric oxide: Regulation and protective and toxic mechanisms. **Current Topics in Cellular Regulation**, 34: 159–187

Winrow, V.R., Winyard, P.G., Morris, C.J., et al. (1993) Free radicals in inflammation: second messengers and mediators of tissue destruction. **British medical bulletin**, 49 (3): 506–22

Zhang, Z., Farrel, A.J., Blake, D.R., Chidwick, K. and Winyard, P.G. (1993) Inactivation of synovial fluid alpha 1-antitrypsin by exercise of the inflamed rheumatoid joint. **Clinical Molecular and Microbiology Papers**, 321 (2-3): 247-278

Chapter Four

4. Impact of low volume high intensity interval exercise on markers of oxidative stress

4.1. Abstract

Purpose: Low volume, high intensity interval training (LV-HIIT) is a time and energy-efficient exercise strategy producing similar beneficial physiological effects to those of steady state exercise. Acute oxidative stress and inflammation are potential mediators of the adaptations to exercise, however the oxidative stress and inflammatory responses to a bout of LV-HIIT are unknown.

Methods: Untrained healthy males ($n=10$, mean \pm SD: age 22 ± 3 yrs; VO_{2MAX} 42.7 ± 5.0 ml/kg/min⁻¹) undertook three exercise bouts: a bout of LV-HIIT (10*1 minute 90% VO_{2MAX} intervals) and two energy-matched steady-state cycling bouts at a moderate (60% VO_{2MAX} ; 27 min, MOD) and high (80% VO_{2MAX} ; 20 min, HIGH) intensity on separate days. Blood samples were taken before (baseline), during the last minute of exercise (exercise) and 30 minutes following exercise (post+30). Markers of oxidative stress and inflammation were assessed, in addition to lymphocyte number, and adrenaline. Blood pressure and heart rate was measured continuously.

Results: HIGH caused the greatest lymphocytosis, adrenaline and cardiovascular response ($p's < .05$). In all exercise bouts, lipid hydroperoxides (LOOH) and total antioxidant capacity (TAC) were significantly increased during and post+30, relative to baseline and during exercise respectively (LOOH (nmol/ml plasma): MOD +0.36; HIGH +3.09; LV-HIIT +5.51 and TAC (μ M): MOD +189.44; HIGH +134.72; LV-HIIT +102.24). IL-6 and IL-10 increased post+30 in HIGH and LV-HIIT only.

Conclusions: HIGH was the greatest physiological stressor of the three bouts of exercise compared in the present study. LV-HIIT was more comparable to HIGH when markers of oxidative stress and inflammation were assessed. LV-HIIT and HIGH, but not MOD stimulated an anti-inflammatory response to exercise.

4.2. Introduction

Reactive oxygen and nitrogen species (RONS) are by products of normal cellular metabolism that regulate cellular signalling and homeostasis. Oxidative stress is a state whereby RONS exceed endogenous and exogenous antioxidants systems, resulting in the progressive oxidation of molecules (proteins, lipids and DNA) within the body. Chronic oxidative stress is widely implicated with the ageing process (Harman, 1956) and a range of human diseases (Aldred, 2007). While exercise training may represent a valuable means of reducing chronic oxidative stress in some populations (Donato et al., 2010), an acute bout of steady state exercise is known to cause oxidative stress (Michailidis et al., 2007). It is now widely accepted that the increase in RONS that follows an acute bout of exercise can facilitate a host of beneficial adaptations within skeletal muscle (Gomez-Cabrera et al., 2008b; Ristow et al., 2009). We recently suggested that there is a reciprocal relationship between oxidative stress and inflammation (Wadley et al., 2012) and there is now strong evidence to suggest that interleukin (IL)-6 can regulate substrate metabolism (Febbraio et al., 2004) and stimulate anti-inflammatory cytokines such as IL-10, following exercise (Fischer, 2006). However, the influence of exercise modality on markers of oxidative stress and inflammation remain incompletely understood.

The most frequent barrier to participation in regular physical exercise within the general population is a perceived 'lack of time' (Stutts, 2002). The American College of Sports Medicine guidelines recommend up to 150 minutes of moderate intensity exercise per week (ACSM, 2005). High intensity interval training (HIIT) is defined as short, intense bursts of physical activity, combined with rest or low intensity intervals (Burgomaster et al., 2008). There is ample evidence to suggest that HIIT is equally effective in improving metabolic (Burgomaster et al., 2008), physiological (Wisløff et al., 2007) and vascular health (Cocks et al., 2012) as traditional steady-state exercise training, but with a reduced time commitment.

Modified HIIT protocols (Low volume HIIT (LV-HIIT)) have been developed for individuals not wishing to, or who are unable to engage in extremely demanding short bursts of high intensity physical activity (Gibala et al., 2012; Wisløff et al., 2007). LV-HIIT has been shown to increase markers of muscle metabolism (Hood et al., 2011) induce increases in VO_2 peak, (Rognmo et al., 2004) and improve endothelial function (Wisløff et al., 2007).

There is some evidence to suggest that the magnitude of increase in RONS (Bailey et al., 2004) and cytokines (Ostrowski et al., 2000) in response to exercise is intensity dependent, and given the role of RONS in exercise adaptation it is important to characterise the magnitude of this response. Recent evidence has indicated that LV-HIIT can induce an increase in plasma lipid oxidation and antioxidant enzyme activity immediately following exercise (Fisher et al., 2011). To our knowledge, there are no studies to date that characterise the response of oxidative stress and inflammation to LV-HIIT in the context of steady state exercise. Further, no studies have compared these changes with other physiological responses (cardiovascular, immune and hormonal markers). The aim of this pilot study was to compare plasma markers of oxidative stress and inflammation in response to LV-HIIT, high and moderate intensity steady state exercise bouts in a small sample of untrained males. Changes in total lymphocyte number, plasma adrenaline, heart rate and blood pressure were also assessed. We tested the hypothesis that LV-HIIT will elicit a significantly greater increase in oxidative stress and inflammation than moderate intensity steady state exercise.

4.3. Methods

4.3.1. Participants

Ten healthy, untrained (defined as $\text{VO}_{2\text{MAX}} < 50 \text{ kg/ml/min}^{-1}$) males (mean \pm SD: age 22 ± 3 yrs; body mass index $24.0 \pm 3.1 \text{ kg/m}^2$; $\text{VO}_{2\text{MAX}} 42.7 \pm 5.0 \text{ ml/kg/min}^{-1}$) took part in three separate exercise bouts. All participants gave their informed written consent and the investigation was approved by the Science and Technology ethical review committee at the University of Birmingham. Participants were non-smokers and excluded if they had ingested vitamin supplements or anti-inflammatory drugs in the two weeks prior to the first laboratory visit. In addition, subjects were required to abstain from foods high in dietary nitrate (beetroot, lettuce, spinach and processed meats) at least two days prior to each experimental session. In addition, participants were required to refrain from any strenuous physical activity or consumption of alcoholic beverages in the 48 hours prior to testing sessions.

4.3.2. Preliminary Assessments

Participants undertook all bouts in the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. All exercise bouts took place on an electromagnetically braked cycle ergometer (*Lode Excalibur Sport*, Groningen, Netherlands). Height and weight was recorded (*Seca Alpha*, Hamburg, Germany) and questionnaires administered for demographic and health screening purposes (Fisher and White, 1999). Cardiorespiratory fitness was assessed by determining the maximum oxygen consumption ($\text{VO}_{2\text{MAX}}$) of the participant and expressed relative to body weight ($\text{ml.kg}^{-1}\text{min}^{-1}$). A breath-by-breath system (*Oxygon Prx*, Jaeger, Wuerzburg, Germany) was used for continuous measurement of oxygen uptake and heart rate was monitored using a Polar Vantage heart rate monitor (Polar Vantage, Kempele, Finland). After a 3 minute warm up at 30 Watts, participants undertook an incremental exercise test to exhaustion, whereby workload

increased 30 Watts every minute until volitional exhaustion. Participants were asked to maintain a constant pedal rate and encouragement was given by an experimenter. A respiratory exchange ratio (VCO_2/VO_2) >1.10-1.15, plateau in participant oxygen consumption or a maximal heart rate >220 beats min^{-1} - age were all factors used to indicate $VO_{2\text{MAX}}$ and thus the termination of the test (Howley et al., 1995).

4.3.3. Exercise Bouts

One week after the first visit, participants returned to the laboratory following an overnight fast to undertake one of three randomised exercise bouts, each separated by at least one week (figure 4.1). Following a 30 minute period of rest, a catheter (*Becton, Dickson & Company, Oxford, UK*) was inserted into the antecubital vein of the forearm and a baseline blood sample taken (baseline). The catheter was kept patent through regular flushes with saline (0.9% NaCl). Participants then undertook a 5 minute warm up at a workload that elicited 40% of their maximum aerobic capacity, followed by the exercise bout at 60% $VO_{2\text{MAX}}$ for 27 minutes (Moderate, MOD), 80% $VO_{2\text{MAX}}$ for 20 minutes (HIGH) and ten 1 minute stages at 90% $VO_{2\text{MAX}}$, interspersed with nine 1 minute intervals at 40 % $VO_{2\text{MAX}}$ (LV-HIIT) (figure 4.1). MOD and HIGH were matched for total workload (kcal). A second blood sample (exercise) was taken during the last minute of each exercise bout to capture the peak physiological response and then 30 minutes following cessation of the exercise bout (post+30). At each time point, 5 ml of blood was drawn into a vacutainer tube containing potassium ethylene diaminetetraacetic acid (*Becton, Dickson & Company, Oxford, UK*) and centrifuged at 2800 rpm for 15 minutes at 4 °C. Plasma was then extracted and stored at -80 °C until further analysis.

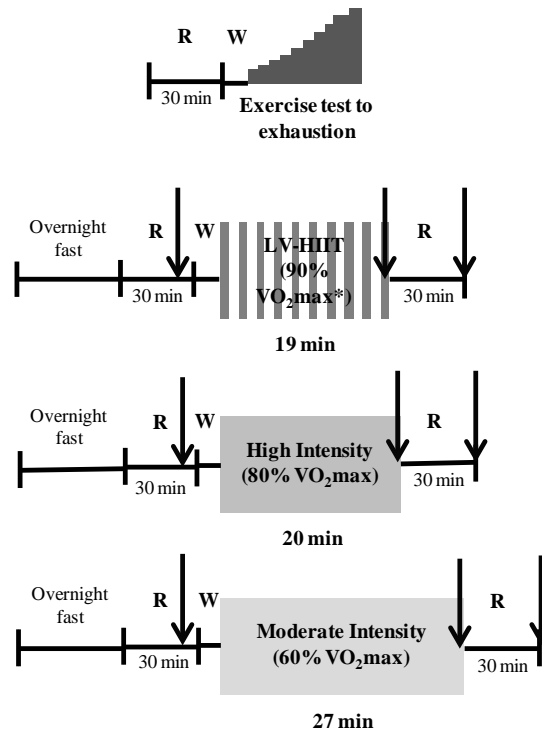


Figure 4.1: Study Protocol. Subjects completed an exercise test to exhaustion and then participated in 3 subsequent exercise bouts. LV-HIIT was ten 1 minute stages at 90% VO₂MAX, interspersed with nine 1 minute intervals at 40% VO₂MAX.

↓ Blood samples (baseline, exercise & post+30); R, Rest; W, Warm up (5min, 40% VO₂MAX)

4.3.4. Blood Assessments

Blood samples were assessed for blood cell composition, specifically total peripheral blood lymphocytes. In addition, haemoglobin (g/dL) and haematocrit (%) were assessed to calculate plasma volume changes as a result of exercise (Bacon et al., 2004) (*Coulter Analyser, Beckman-Coulter, High Wycombe, UK*). All reagents purchased from Sigma unless stated (*Sigma Aldrich, Dorset, UK*).

Total Antioxidant Capacity

TAC was assessed using the Ferric Reducing Ability of Plasma (FRAP) assay (Benzie and Strain, 1996). Plasma samples (10 μ l per well) and standards (ascorbic acid, 0-1000 μ M; 10 μ l) were added in triplicate to a flat bottomed 96 well plate. FRAP reagent (300 mM sodium acetate (pH=3.6), 160 mM 2, 4, 6- tripyridyltriazin and 20mM ferric chloride (FeCl_3); 300 μ l) was added to each well and left to incubate for 8 minutes at room temperature, then absorbance's read at 650 nm. TAC values were obtained using absorbance values of known ascorbic acid concentrations, expressed as μ M of antioxidant power relative to ascorbic acid (McAnulty et al., 2005) and adjusted for changes in plasma volume. The inter and intra-assay coefficients of variation (CV) are <3% and <1% respectively (Benzie and Strain, 1996).

Lipid Hydroperoxides

LOOH concentrations were assessed using a spectrophotometric assay (Görög et al., 1991). Samples and a blank standard (10 μ l) were added to a 96 well microtitre plate in triplicate. Reagent mix (100 μ l, 0.2 M Potassium phosphate (pH=6.2), 0.12 M potassium iodide, 0.15 mM sodium azide, Triton X (2 g/l), alkylbenzyltrimethylammonium (0.1 g/l), 10 μ M ammonium molybdate and HPLC grade water (to make total volume 100 ml)) was added for 30 minutes at room temperature, away from light on a plate shaker. The plate was read at 340nm (Multiscan MS, Labsystems), concentration of lipid peroxides (μ mol/l) determined using the Beer-Lambert Law (extinction co-efficient $\epsilon_{340} = 24600 \text{ M}^{-1}\text{cm}^{-1}$) and adjusted for changes in plasma volume. The inter-assay CV is 8.9% (El-Saadani et al, 1989).

Nitrite and Nitrate (NOx metabolites)

The Griess assay was used to quantify total nitric oxide metabolites (NOx) in plasma samples (Miranda et al., 2001). Samples (100 μ l) were diluted with HPLC grade water (375

µl) and zinc sulphate (25 µl, 300 mg/ml) to give a concentration of approximately 15 mg/ml. Samples were vortexed and then centrifuged at 10,000 g for 20 minutes (10 °C). Supernatants and standards (100 µl) were added to a 96 well microtitre plate in triplicate, and 100 µl vanadium (III) chloride (8 mg/ml) then added. Thorough mixing was ensured to fully reduce plasma nitrate to nitrite. Sulphanilamide (50 µl, 2 %) and N-(1-naphthyl) ethylenediamine dihydrochloride (50 µl, 0.1 %) were then rapidly added to each well. Plates were incubated for 30 minutes at 37 °C, absorbance read at 540 nm and compared with values of known nitrite concentrations (0-100 µM). Values were adjusted for changes in plasma volume. The inter and intra-assay CVs are 5.2% and 4.4% respectively (Asl et al, 2008).

Interleukins

Plasma concentrations of IL-6 were determined using a commercially available high-sensitivity (HS) ELISA kit according to manufacturer instructions (*R&D Systems*, assay sensitivity: 0.11 pg/ml). Briefly, samples (200 µl, diluted 1:1 with assay diluent) were incubated with a monoclonal antibody (200 µl) derived against IL-6, and then substrate (50 µl) and amplifier (50 µl) solutions added to develop the colour change. Plates were washed intermittently to reduce non-specific binding. The reaction was stopped using 2 M sulphuric acid (50 µl) and absorbance's read at 650nm. Values were then obtained from a linear standard curve of known IL-6 concentrations (0.156-10 pg/ml) and adjusted for changes in plasma volume.

Plasma concentrations of IL-10 were determined using a commercially available HS ELISA kit, according to manufacturer instructions (*R&D Systems*, assay sensitivity: 0.17 pg/ml). Briefly, samples (250 µl, diluted 4:1 with assay diluent) were incubated with a monoclonal antibody (200 µl) derived against IL-10 and then substrate (50 µl) and amplifier (50 µl) solutions added to develop a colour change. Plates were washed intermittently to

reduce non-specific binding. The reaction was stopped using 2 M sulphuric acid (50 µl) and absorbance read at 490 nm. Values were then obtained from a linear standard curve of known IL-10 concentrations (0.78-50 pg/ml) and adjusted for changes in plasma volume. The inter and intra-assay CVs for HS IL-6 and IL-10 kits are 6.5% and 6.9% respectively (*R&D Systems*).

Adrenaline

Plasma concentrations of adrenaline were determined using a commercially available High-Sensitivity ELISA kit (assay sensitivity: 3 pg/ml), according to manufacturer instructions (*Rocky Mountain Diagnostics Inc. USA*). Briefly, adrenaline was extracted from plasma samples (750 µl) using a cis-diol-specific affinity gel, acylated and then derivitised enzymatically. Extracted plasma samples and controls (100 µl) were incubated with a monoclonal antibody (50 µl) derived against adrenaline and incubated overnight at 4 °C. The plate was then incubated with an enzyme conjugate (100 µl) and substrate (100 µl) to develop the colour change. Plates were washed intermittently to reduce non-specific binding. The reaction was stopped using 0.25 M sulphuric acid (100 µl) and absorbance's read at 450 nm. Values were obtained from a standard curve of known adrenaline concentrations (0-1500 pg/ml) and adjusted for changes in plasma volume. The intra-assay CV is 9.3% (*Rocky Mountain Diagnostics Inc. USA*).

4.3.5. Sample size calculation and Statistical Analysis

Power analyses using Gpower3 (Faul et al., 2007), with significance at .05 and power at .90, were conducted based upon results from previous studies and preliminary pilot work assessing markers of inflammation and oxidative stress following steady state exercise in sedentary young adults. Outcome measures of protein oxidation, IL-6 concentration changes

(2-fold) were used, based on previous studies from our laboratory. A sample size of 10 participants was required to detect differences with an effect size of .24 (medium effect size).

Statistical analyses were performed using SPSS (PASW Statistics, release 21.0, SPSS Inc., Chicago, IL, USA). Kolmogorov–Smirnov tests were used to investigate normal distribution and differences between variables at baseline were assessed using one-way analyses of variance (ANOVA). The physiological response to exercise was assessed by a 3 exercise bout (MOD, HIGH, LV-HIIT) by 3 time (baseline, exercise, post+30) repeated-measures ANOVA. Post hoc analysis of the interaction effects was performed by a test of simple effects by pairwise comparisons, with Bonferroni correction. Assessments of heart rate and blood pressure over time by area under the curve (AUC) were undertaken using one-way repeated measures ANOVA. Pearson correlations were conducted to examine associations between adrenaline and total lymphocyte count. These analyses were performed separately for LV-HIIT and steady state bouts. Statistical significance was accepted at the $p < 0.05$ level.

4.4. Results

4.4.1. Oxidative Stress

Lipid Hydroperoxides

Figure 4.2A shows the response of plasma LOOH to the three different exercise bouts. LOOH significantly increased during exercise in all bouts ($p=0.033$). Thirty minutes following exercise, values had returned to baseline concentrations ($p=0.023$). No statistical differences were observed between bouts. Data was not normally distributed and subsequently log transformed prior to statistical analyses (Log10).

Total Antioxidant Capacity

Figure 4.2B shows the response of plasma TAC to the different exercise bouts. A trend was observed for a decrease in TAC during exercise in all bouts, however this did not reach statistical significance ($p=0.161$). Thirty minutes after exercise, TAC was significantly higher than during exercise (pairwise comparisons) in all bouts ($p=0.004$). No statistical differences were observed between bouts.

4.4.2. Inflammation

Interleukins

The effect of exercise on IL-6 concentrations can be seen in Figure 4.2C. IL-6 increased during exercise ($p=0.05$) and post+30 ($p=0.001$) in all bouts. A significant group x time interaction effect was found ($p=0.012$) and pairwise comparisons indicated that the concentrations of IL-6 post+30 were significantly higher in HIGH compared to MOD ($p=0.037$). There were statistically significant increases in IL-6 post+30, relative to baseline (LV-HIIT: $p=0.010$; HIGH: $p=0.016$) and exercise (LV-HIIT: $p=0.029$; HIGH: $p=0.048$) in

LV-HIIT and HIGH. IL-6 increased post+30, relative to baseline in MOD ($p=0.10$). There were no statistical differences between the responses in LV-HIIT and HIGH.

Figure 4.2D shows the response of IL-10 to the different exercise bouts. IL-10 was unchanged during exercise, however a significant increase was observed post+30 in HIGH and LV-HIIT (main effect of time: $p=0.042$). A significant group x time interaction effect was found ($p=0.015$) and pairwise comparisons indicated that the concentrations of IL-10 post+30 were higher in HIGH than MOD ($p=0.05$). IL-10 increased post+30 relative to baseline ($p=0.05$) and during exercise ($p=0.05$) in LV-HIIT and HIGH respectively. There were no statistical differences between the response of LV-HIIT and HIGH.

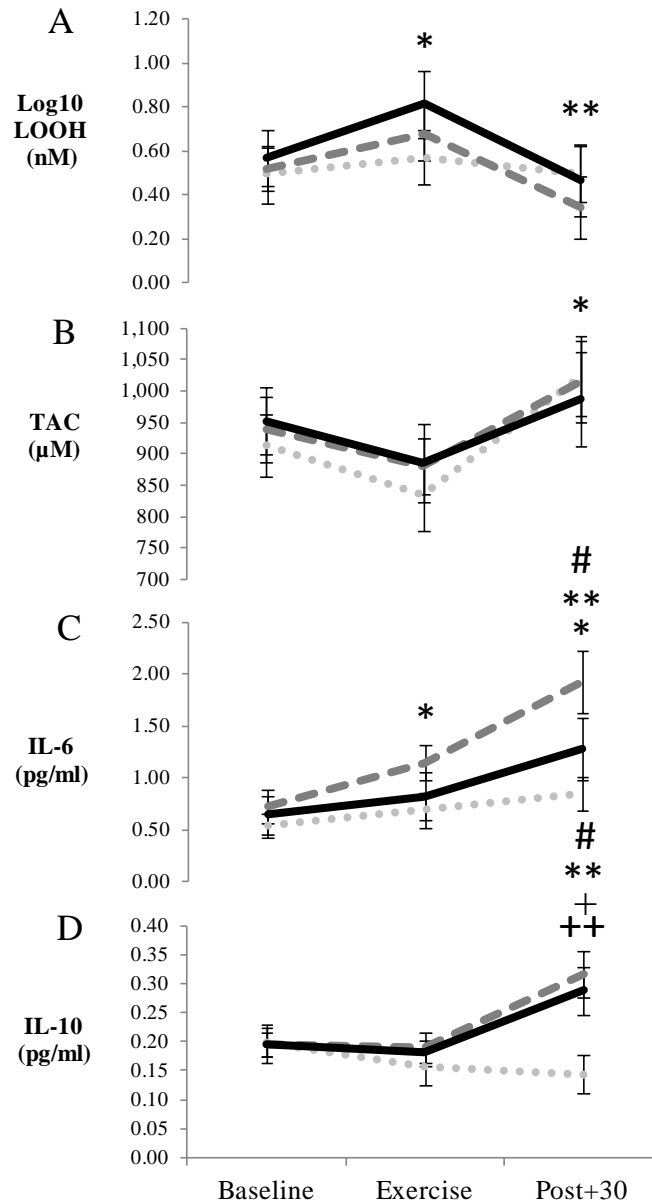


Figure 4.2: Changes in oxidative stress and inflammation in response to the three exercise bouts (n=10). (A) LOOH (B) TAC (C) IL-6 and (D) IL-10. Values are means \pm standard error. * indicates significant differences during exercise, relative to baseline in all bouts ($p < 0.05$). ** indicates significant differences relative to during exercise in all bouts. # indicates significant differences between HIGH and MOD. Figure 2D: + indicates a significant difference between baseline and post+30 in LV-HIIT. ++ indicates a significant differences between exercise and post+30 in HIGH. MOD (.....), HIGH (---) and LV-HIIT (—).

4.4.3. Other physiological measures

Total Peripheral Blood Lymphocytes

Figure 4.3A shows the response of total peripheral blood lymphocytes to the different exercise bouts. There was a significant lymphocytosis during exercise in all bouts ($p < 0.001$), which returned to baseline values post+30 ($p < 0.001$). A significant group x time interaction effect was found ($p < 0.001$) and pairwise comparisons indicated that HIGH elicited a significantly greater lymphocytosis than both LV-HIIT ($p = 0.032$) and MOD ($p = 0.002$) during exercise. There were no statistical differences between the response during LV-HIIT and MOD.

Adrenaline

The effect of exercise on adrenaline concentrations can be seen in figure 4.3B. Adrenaline significantly increased during exercise in all bouts ($p = 0.04$) and returned to baseline values post+30 ($p = 0.10$). A significant group x time interaction effect was found ($p < 0.001$) and pairwise comparisons indicated that HIGH elicited a significantly greater adrenaline response than both LV-HIIT ($p = 0.049$) and MOD ($p = 0.037$) during exercise. There were no statistical differences between the responses during exercise in LV-HIIT and MOD.

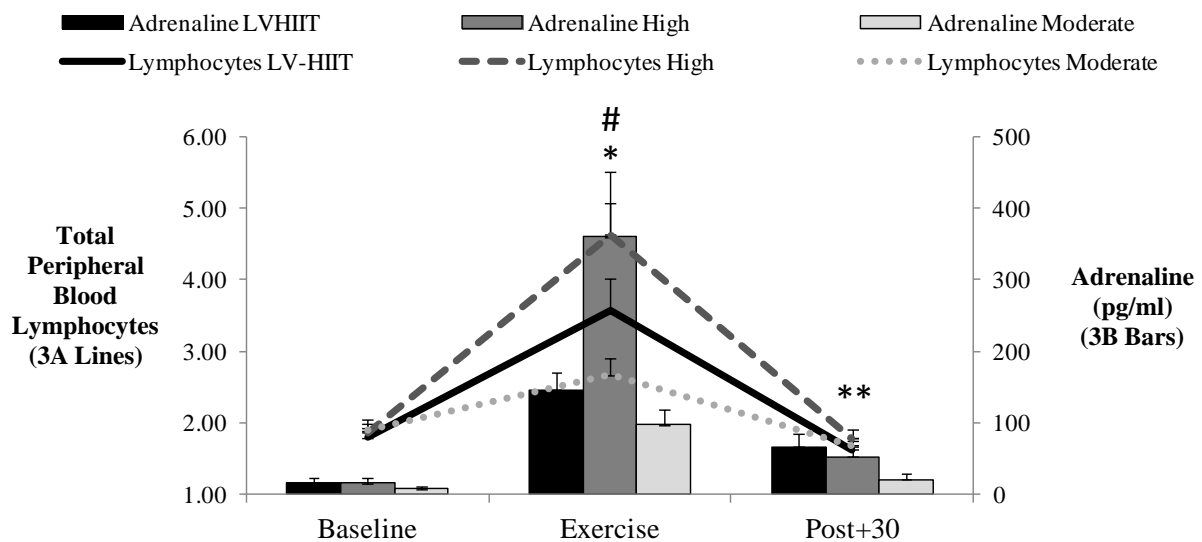


Figure 4.3: Changes in total lymphocyte number (A) and plasma adrenaline (B) in response to the three exercise bouts (n=10). Values are means \pm standard error. * indicates significant differences during exercise, relative to baseline ($p < 0.05$). ** indicates significant differences relative to during exercise. # indicates significant differences compared to MOD and LV-HIIT.

Heart Rate and Blood Pressure

The effect of exercise on baseline to peak heart rate, systolic blood pressure (SBP) and diastolic blood pressure (DBP) during the exercise bouts is shown in figure 4.4. There was a significant increase in heart rate during exercise in all bouts ($p < 0.001$), which returned to baseline values post+30 ($p < 0.001$). A significant group x time interaction effect was found ($p < 0.001$) and pairwise comparisons indicated that HIGH elicited a significantly greater heart rate response than MOD ($p < 0.001$) during exercise. The magnitude of heart rate change during HIGH compared to LV-HIIT did not reach statistical significance during exercise ($p = 0.074$). Thirty minutes following exercise, heart rate in both the LV-HIIT ($p = 0.001$) and HIGH ($p = 0.002$) was greater than MOD.

There was a significant increase in SBP during exercise in all bouts ($p<0.001$), which returned to baseline values post+30 ($p=0.01$). A significant group x time interaction effect was found ($p=0.04$) and pairwise comparisons indicated that LV-HIIT elicited a significantly greater peak SBP response than MOD ($p=0.037$). No differences were observed between the SBP response during HIGH and LV-HIIT. The SBP response thirty minutes following exercise in HIGH was significantly lower than MOD ($p=0.003$).

Figure 4.5 indicates the changes in heart rate and blood pressure over time as assessed by the area under the curve (AUC). Total AUC for heart rate was significantly lower in MOD and LV-HIIT when compared to HIGH (MOD -31.8% ($p<0.001$) and LV-HIIT -31.1% ($p=0.001$)). A similar trend was observed for SBP, however only LV-HIIT was significantly lower than HIGH (MOD -33.5% ($p=0.098$) and LV-HIIT -29.1% ($p=0.034$)) (Figure 4.5).

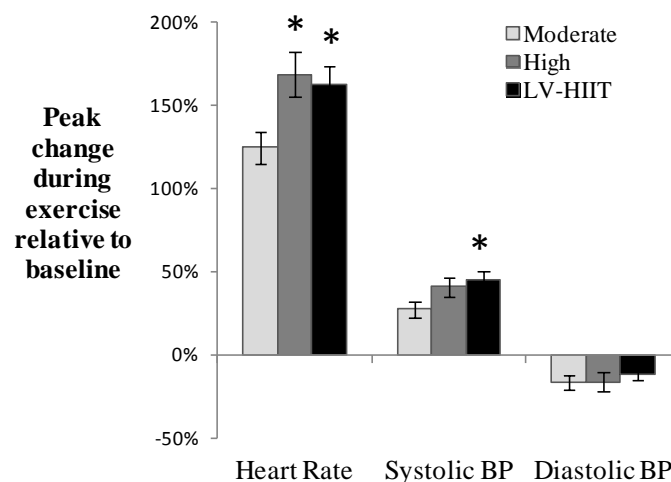


Figure 4.4: Cardiovascular response during the three exercise bouts ($n=10$). The percentage change in heart rate, systolic blood pressure and diastolic blood pressure from baseline to during the exercise bouts. Data are means \pm standard error. * indicates significant differences relative to moderate bout ($p<0.05$).

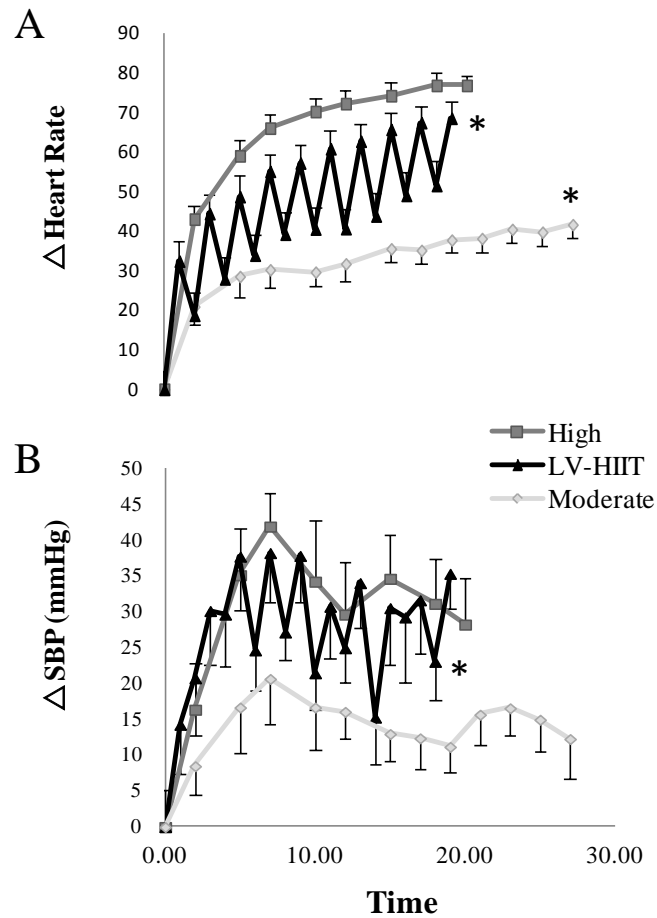


Figure 4.5: Area under the curve analysis of the cardiovascular response during the three exercise bouts (n=10). The change in heart rate (5A) and systolic blood pressure (5B) relative to baseline are shown. Data are means \pm standard error. * indicates significant differences relative to HIGH ($p<0.05$).

Nitric Oxide Metabolites

Table 4.1 shows the response of plasma NOx to the different exercise bouts. There was a trend towards an increase in NOx both during exercise and post+30 in LV-HIIT only however this did not reach statistical significance.

Table 4.1: NO_x concentrations in response to the three exercise bouts (n=10). Values are means \pm standard error.

	Moderate			High			LV-HIIT		
	Baseline	Exercise	Post+30	Baseline	Exercise	Post+30	Baseline	Exercise	Post+30
NO_x	17.77	16.95	17.73	23.20	23.76	31.41	31.16	39.58	49.19
(μ M)	(± 3.02)	(± 2.55)	(± 4.15)	(± 7.90)	(± 6.57)	(± 8.17)	(± 5.32)	(± 7.96)	(± 11.36)

4.4.4. Correlation Analysis

Correlation analysis indicated a significant positive correlation between the change in adrenaline and total lymphocyte count between baseline and during exercise in both HIGH ($r=0.681$, $p=0.015$) and LV-HIIT ($r=0.851$, $p=0.001$) bouts. No significant correlations were observed between the change in NO_x and total lymphocyte count between baseline and during exercise in any of the bouts.

4.5. Discussion

To our knowledge this is the first study to assess oxidative stress following a bout of LV-HIIT in comparison to steady state exercise. Our pilot findings are that LV-HIIT and steady state exercise caused a significant increase in markers of oxidative stress and inflammation. LV-HIIT was more comparable to HIGH than MOD when the magnitude of change in markers of oxidative stress was considered. The significant increases in LOOH concentrations observed in all bouts do indicate the presence of RONS following exercise.

With compelling evidence that exercise-induced increases in oxidative stress are adaptive (Gomez-Cabrera et al., 2008a; Ristow et al., 2009), the peroxidation of lipids may stimulate adaptations such as structural remodelling of external cellular membranes and lipoproteins. The observed antioxidant response to all bouts of exercise is in agreement with previous studies (Berzosa et al., 2011; Turner et al., 2011). An increase in uric acid production from purine degradation (Cao and Prior, 1998), increases in vitamin C release from the adrenal glands (Gleeson, M., Robertson, J.D., Maughan, 1987) and an influx of cells with high cell-surface thiol content (Turner et al., 2011) could all be mechanisms behind this response. Collectively, these data are the first to indicate the LV-HIIT elicits a comparable oxidative stress response to steady state exercise. This pilot data could be used to power future larger-scale studies.

IL-6 and IL-10 significantly increased in response to LV-HIIT and HIGH, indicating these exercise bouts were more effective than MOD in stimulating an anti-inflammatory response to exercise. Muscle contraction (Petersen et al., 2001), substrate availability (Febbraio et al., 2004) and adrenaline release (Steensberg et al., 2001) have all been linked with IL-6 transcription regulation and release into the circulation in response to exercise. Aside from the classical role of IL-6 to signal and facilitate the inflammatory response, there is also evidence to suggest that its release during exercise can regulate glucose uptake into

skeletal muscle (Febbraio et al., 2004) and transcription of anti-inflammatory cytokines such as IL-10 (Fischer, 2006). The present results are in support of these studies, with IL-10 elevation seen thirty minutes following the increase in IL-6 in both LV-HIIT and HIGH. In the current study the largest IL-6 and IL-10 responses following exercise were in response to HIGH. Previous evidence has highlighted that exercise intensity may be the key factor governing IL-6 release when considering shorter bouts of steady state exercise under an hour (Ostrowski et al., 2000; Fischer, 2006). However, no studies to date have assessed the cytokine response to a bout of LV-HIIT. IL-6 and IL-10 increased following LV-HIIT, with the magnitude of this response appearing to sit in-between the two steady state bouts. Despite the higher peak intensity of LV-HIIT, the intermittent nature of the stimulus could have aided the clearance of both cytokines by the liver and kidneys (Febbraio et al., 2003) during the 'rest' intervals. Nevertheless, these data indicate that LV-HIIT and short duration high intensity, but not moderate intensity steady state exercise, can elicit an anti-inflammatory response in untrained subjects. This is encouraging if the use of LV-HIIT is considered in patient populations given the reduced energy cost compared to steady state training.

These data show that HIGH caused a significantly greater lymphocytosis and adrenaline response than MOD and LV-HIIT. Peak heart rate and SBP values were not different between LV-HIIT and HIGH. However, when changes in heart rate and SBP over time (AUC) were assessed as previously demonstrated (Fisher and White, 1999), LV-HIIT was more similar to MOD (figure 4.5). In line with previous work, intensity dependent increases in plasma adrenaline and total circulating lymphocyte number have regularly been observed (Krüger et al., 2008). However, despite higher peak intensity, LV-HIIT caused a lower adrenaline response and lymphocytosis than HIGH. Previous studies have observed up to a 35% drop in plasma adrenaline just 1 minute after cessation of the exercise stimulus (Zouhal et al., 2008), therefore, it may be possible that the lower response is due to clearance

of adrenaline in-between intervals. The correlation observed between the change in adrenaline and total lymphocyte count in response to exercise in both HIGH ($r=0.681$, $p=0.015$) and LV-HIIT ($r=0.851$, $p=0.001$) supports previous evidence that adrenaline can mobilise lymphocytes off the endothelial wall in response to exercise (Madden et al., 1995). Given that no differences in the response of NOx were observed in any of the exercise bouts (table 4.1), it appears that in this study, lymphocyte mobilisation was related to adrenaline release.

In conclusion, this study presents evidence to suggest that a bout of LV-HIIT does not stimulate a significantly different response in markers of oxidative stress to the responses seen following short duration high or moderate intensity steady state exercise. When considering other physiological markers (i.e. heart rate, adrenaline and lymphocytosis), HIGH was the greatest stressor, with LV-HIIT being more comparable to MOD. When assessing the anti-inflammatory response to exercise, LV-HIIT was more comparable to HIGH.

4.6. Reference list for chapter four

ACSM (2005) "Guidelines for exercise testing and prescription." In **7th ed. Philadelphia, Pa: Lippincott Williams & Wilkins**. pp. 286–299

Aldred, S. (2007) Oxidative and nitrative changes seen in lipoproteins following exercise. **Atherosclerosis**, 192 (1): 1–8

Asl, S.Z., Ghasemi, A. and Azizi, F. (2008). Serum nitric oxide metabolites in subjects with metabolic syndrome. **Clinical Biochemistry**, 41: 1342-1347.

Bacon, S.L., Ring, C., Lip, G.Y.H., et al. (2004) Increases in lipids and immune cells in response to exercise and mental stress in patients with suspected coronary artery disease: effects of adjustment for shifts in plasma volume. **Biological Psychology**, 65 (3): 237–250

Bailey, D.M., Young, I.S., McEneny, J., et al. (2004) Regulation of free radical outflow from an isolated muscle bed in exercising humans. **American Journal of Physiology-Heart and Circulatory Physiology**, 287 (4): H1689–H1699

Benzie, I.F. and Strain, J.J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. **Analytical Biochemistry**, 239 (1): 70–76

Berzosa, C., Cebrian, I., Fuentes-Broto, L., et al. (2011) Acute exercise increases plasma total antioxidant status and antioxidant enzyme activities in untrained men. **J Biomed Biotechnol**, pp. 1–7

Burgomaster, K.A., Howarth, K.R., Phillips, S.M., et al. (2008) Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. **Journal of Physiology**, 1: 151–160

Cao, G. and Prior, R.L. (1998) Comparison of different analytical methods for assessing total antioxidant capacity of human serum. **Clinical chemistry**, 44 (6 Pt 1): 1309–15

Cocks, M., Shaw, C.S., Shepherd, S.O., et al. (2012) High intensity interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males. **The Journal of Physiology**, pp. 1–43

Donato, A.J., Uberoi, A., Bailey, D.M., et al. (2010) Exercise-induced brachial artery vasodilation: effects of antioxidants and exercise training in elderly men. **Am J Physiol Heart Circ Physiol**, 298 (2): H671–8

El-Saadani, M., Esterbauer, H., El-Sayed, M., et al. (1989) A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. **Journal of Lipid Research**, 30 (4): 627–630

Faul, F., Erdfelder, E., Lang, A.G., et al. (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. **Behavior research methods**, 39 (2): 175–91

Febbraio, M.A., Hiscock, N., Sacchetti, M., et al. (2004) Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction. **Diabetes**, 53 (7): 1643–8

Febbraio, M.A., Ott, P., Nielsen, H.B., et al. (2003) Hepatosplanchnic clearance of interleukin-6 in humans during exercise. **American journal of physiology Endocrinology and metabolism**, 285 (2): E397–402

Fischer, C.P. (2006) Interleukin-6 in acute exercise and training: what is the biological relevance? **Exercise immunology review**, 12 (115): 6–33

Fisher, G., Pascoe, D.D., Schwartz, D.D., et al. (2011) Lymphocyte enzymatic antioxidant responses to oxidative stress following high-intensity interval exercise Lymphocyte enzymatic antioxidant responses to oxidative stress following high-intensity interval exercise. **Journal of Applied Physiology**, 110: 730–737

Fisher, W.J. and White, M.J. (1999) Training-induced adaptations in the central command and peripheral reflex components of the pressor response to isometric exercise of the human triceps surae. **The Journal of physiology**, 520 Pt 2: 621–8

Gibala, M.J., Little, J.P., MacDonald, M.J., et al. (2012) Physiological adaptations to low-volume, high-intensity interval training in health and disease. **Journal of Physiology**, 590.5: 1077–1084

Gleeson, M., Robertson, J.D., Maughan, R.J. (1987) Influence of exercise on ascorbic acid status in man. **Clin Sci (Lond)**, 73 (5): 501–505

Gomez-Cabrera, M.C., Domenech, E., Romagnoli, M., et al. (2008a) Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. **The American journal of clinical nutrition**, 87 (1): 142–9

Gomez-Cabrera, M.C., Domenech, E. and Vina, J. (2008b) Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. **Free Radic Biol Med**, 44 (2): 126–131

Görög, P., Kotak, D.C. and Kovacs, I.B. (1991) Simple and specific test for measuring lipid peroxides in plasma. **Journal of clinical pathology**, 44 (9): 765–7

Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry. **J Gerontol**, 11 (3): 298–300

Hood, M.S., Little, J.P., Tarnopolsky, M.A., et al. (2011) Low-volume interval training improves muscle oxidative capacity in sedentary adults. **Medicine and science in sports and exercise**, 43 (10): 1849–56

Howley, E.T., Bassett Jr, D.R. and Welch, H.G. (1995) Criteria for maximal oxygen uptake: a review and commentary. **Medicine & Science in Sports & Exercise**, 27 (9): 1292–1301

Krüger, K., Lechtermann, A., Fobker, M., et al. (2008) Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. **Brain, behavior, and immunity**, 22 (3): 324–38

Madden, K.S., Sanders, V.M. and Felten, D.L. (1995) Catecholamine influences and sympathetic neural modulation of immune responsiveness. **Annual Reviews Pharmacology Toxicology**, 35: 417–448

McAnulty, S.R., McAnulty, L.S., Morrow, J.D., et al. (2005) Effect of daily fruit ingestion on angiotensin converting enzyme activity, blood pressure, and oxidative stress in chronic smokers. **Free Radic Res**, 39 (11): 1241–1248

Michailidis, Y., Jamurtas, A.Z., Nikolaidis, M.G., et al. (2007) Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress. **Medicine and science in sports and exercise**, 39 (7): 1107–13

Miranda, K.M., Espey, M.G. and Wink, D.A. (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. **Nitric Oxide**, 5 (1): 62–71

Ostrowski, K., Schjerling, P. and Pedersen, B.K. (2000) Physical activity and plasma interleukin-6 in humans effect of intensity of exercise. **European journal of applied physiology**, 83 (6): 512–5

Petersen, E.W., Ostrowski, K., Ibfelt, T., et al. (2001) Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise. **Am J Physiol Cell Physiol**, 280: C1570–C1575

Ristow, M., Birringer, M., Kiehntopf, M., et al. (2009) Antioxidants prevent health-promoting effects. **PNAS**, 106 (21): 8865–8670

Rognmo, Ø., Hetland, E., Helgerud, J., et al. (2004) High intensity aerobic interval exercise is superior to moderate intensity exercise for increasing aerobic capacity in patients with coronary artery disease. **European Journal of Cardiovascular Prevention & Rehabilitation**, 11: 216–222

Steensberg, A., Toft, A.D., Schjerling, P., et al. (2001) Plasma interleukin-6 during strenuous exercise : role of epinephrine Plasma interleukin-6 during strenuous exercise : role of epinephrine. **Am J Physiol Cell Physiol**, 281: C1001–C1004

Stutts, W.C. (2002) Physical activity determinants in adults : Perceived benefits , barriers , and self-efficacy. **AAOHN Journal**, 50 (11): 499–507

Turner, J.E., Bosch, J.A., Drayson, M.T., et al. (2011) Assessment of oxidative stress in lymphocytes with exercise. **Journal of applied physiology (Bethesda, Md. : 1985)**, 111 (1): 206–11

Wadley, A.J., Veldhuijzen van Zanten, J.J.C.S. and Aldred, S. (2012) The interactions of oxidative stress and inflammation with vascular dysfunction in ageing : the vascular health triad. **AGE**, 35 (3): 705–718

Wisløff, U., Støylen, A., Loennechen, J.P., et al. (2007) Superior Cardiovascular Effect of Aerobic Interval Training Versus Moderate Continuous Training in Heart. **Circulation**, 115: 3086–3094

Zouhal, H., Jacob, C., Delamarche, P., et al. (2008) Catecholamines and the effects of exercise, training and gender. **Sports medicine (Auckland, N.Z.)**, 38 (5): 401–23

Chapter Five

5. Thioredoxin and over-oxidised peroxiredoxin are increased in peripheral blood mononuclear cells during exercise in humans

5.1. Abstract

Introduction: Peroxiredoxin (PRDX) and Thioredoxin (TRX) are antioxidant proteins that control cellular signalling and redox balance, and are also associated with heightened inflammation. This study aimed to assess key aspects of the PRDX-TRX redox cycle in response to three different modes of exercise.

Methods: Healthy males (n=10, mean \pm SD: age 22 ± 3 yrs) undertook three exercise trials on separate days: two steady-state cycling trials at a moderate (60% $\text{VO}_{2\text{MAX}}$; 27 min, MOD) and high (80% $\text{VO}_{2\text{MAX}}$; 20 min, HIGH) intensity, and a low volume high intensity interval training trial (10*1 min 90% $\text{VO}_{2\text{MAX}}$, LV-HIIT). Peripheral blood mononuclear cells were assessed for TRX reductase (TRX-R) activity, TRX-1 and over-oxidised PRDX (isoforms I-IV) protein expression and plasma for interleukins (IL)-6 and IL-10 before, during and 30 minutes following exercise (post+30).

Results: TRX-1 protein expression increased during exercise in all trials (MOD +84.5%; HIGH +64.1%; LV-HIIT +205.7%; $p < .05$), whereas over-oxidised PRDX increased during HIGH only (MOD -28.7%; HIGH +202.9%; LV-HIIT -22.7%; $p < .05$). TRX-R activity increased during exercise in all trials, with the greatest response seen in HIGH ($p < .05$). IL-6 and IL-10 increased during exercise and post+30, and post+30 only respectively in HIGH and LV-HIIT ($p < .05$).

Discussion: All exercise trials stimulated an increase in TRX-1 protein expression and TRX-R activity. PRDX over-oxidation increased in HIGH only, suggesting that the capacity for TRX to recycle over-oxidised PRDX was reduced compared to MOD and LV-HIIT. Over-oxidation of PRDX was associated with increases in IL-6, adding support for the links between redox proteins and inflammation.

5.2. Introduction

Exercise induces the production of reactive oxygen and nitrogen species (RONS), which act as important signalling molecules in the vast array of metabolic adaptations that take place in human tissues (Gomez-Cabrera et al., 2008; Radak et al., 2005). However, some exercise modes and intensities are also associated with acute increases in cellular oxidative stress, a state whereby RONS overwhelm endogenous antioxidant defence systems (Turner et al., 2011; Sureda et al., 2005). The exercise conditions required to achieve an optimal production of RONS in order to stimulate adaptive processes, versus RONS that may initiate damage, is currently unknown. Changes in markers of oxidative stress with exercise are commonly studied in peripheral blood mononuclear cells (PBMCs) (Tauler et al., 2006; Sureda et al., 2005) and recent work has focussed on antioxidant redox proteins such as Peroxiredoxin (PRDX) (Turner et al., 2013) and Thioredoxin (TRX) (Sumida et al., 2004). PBMCs consist of lymphocytes and monocytes that have various roles in immunity. It has been proposed that a lower antioxidant content within these cells could adversely affect cell function (Messina and Lawrence, 1989). Conversely, abnormally high TRX in PBMCs has been linked with the inhibition of key apoptotic pathways that control the clearance of inflammatory cells (Holmgren and Lu, 2010; Szabó-Taylor et al., 2012). Similarly, both PRDX (Shichita et al., 2012) and TRX (Maurice et al., 1999; Schenk et al., 1996) have been associated with heightened inflammatory cytokine production. Given that oxidative stress and inflammation are associated with a wide variety of metabolic, neurological and inflammatory diseases (Aldred, 2007), understanding how PRDX and TRX regulate the levels of RONS within PBMCs may be essential in aiding understanding of the adaptive and damaging roles of RONS in response to exercise.

PRDX and TRX are ubiquitous oxidoreductase proteins that contain thiol groups with a high capacity to control cellular levels of RONS and reduce oxidative stress (Schenk et al.,

1994; Wood et al., 2003). PRDX directly targets and reduces biological peroxides such as hydrogen peroxide, peroxynitrite and hydroperoxides (Wood et al., 2003) to regulate cellular signalling within lipid rafts (Rhee et al., 2012). TRX is central in maintaining the reduced state of various antioxidant peroxidase enzymes (Burke-Gaffney et al., 2005), including four isoforms (I-IV) of PRDX (Peskin et al., 2007; Kang, 1998). In addition, there is evidence that TRX can directly scavenge hydrogen peroxide (Kang, 1998).

Due to their role in controlling levels of RONS, the oxidation states of TRX and PRDX have been studied extensively to facilitate the understanding of cellular signalling in health and disease. The catalytic cysteine of monomeric PRDX (20-30 kDa) can become oxidised by a peroxidase substrate to form sulfenic acid, before rapidly reacting with an adjacent PRDX molecule to form a dimeric structure (chapter one, figure 1.4). PRDX (isoforms I-IV) redox cycles are known to be predominantly regulated by the TRX redox cycle (Peskin et al., 2007; Rhee et al., 2005). TRX exists in two distinct states; a reduced state (12-14 kDa), with exposed thiol groups, or an oxidised state ($\approx 62-64$ kDa), whereby the thiol groups join to form a disulphide bond that inhibits the antioxidant function of TRX (Zhou et al., 2010; Spindel et al., 2011). Under conditions of high peroxide exposure, PRDX has the capacity to become over-oxidised (Rhee et al., 2007), and exceed the regulatory control of TRX (Low et al., 2008). Over-oxidation of PRDX forms sulfinic and sulfonic acid PRDX oxidation states (PRDX-SO₂₋₃) that have limited or no peroxidase activity respectively (Brinkmann and Brixius, 2013).

Only one study has previously addressed exercise induced changes to PBMC PRDX oxidation states in humans (Turner et al., 2013). Turner et al, (2013) observed an increase in PRDX protein expression and oxidation immediately following an ultra-endurance race (126.7 - 233.4km; 20.4 - 41.4 hours of continuous running) in middle aged men. These data indicated an environment of oxidative stress. The role of TRX in this redox cycle has not

been previously monitored. The only study that has assessed TRX in humans reported an increase in plasma TRX in response to an ultra-endurance race (Marumoto et al., 2010).

To our knowledge no studies have monitored the over-oxidation of PBMC PRDX isoforms (I-IV) and their associations with TRX and inflammation in response to modes of exercise that are more commonly undertaken (i.e. <30 minutes). Further, no studies have examined the impact of exercise intensity on these redox processes. The aim of the present study was to investigate PRDX-1 and TRX-1 protein expression, TRX reductase enzyme activity and the degree of PRDX over-oxidation of PBMCs in response to three short duration exercise trials. In addition, systemic changes in plasma interleukins (IL)-6 and IL-10 were measured.

5.3. Methods

5.3.1. Participants

Ten healthy males (mean \pm SD: age 22 ± 3 yrs; $\text{VO}_{2\text{MAX}}$ 42.7 ± 5.0 ml/kg/min⁻¹) undertook three exercise trials, each separated by at least seven days (figure 5.1). All participants gave their informed written consent and the study was approved by the Science and Technology Ethical Review Committee at the University of Birmingham. No participants had taken any vitamin supplements or anti-inflammatory drugs for fourteen days prior to the first laboratory visit. Subjects were also required to refrain from any strenuous physical activity, consumption of alcoholic beverages or food or drink with high nitrate content (beetroot, lettuce, spinach and processed meats) for at least two days prior to each experimental session.

5.3.2. Preliminary Assessments

All experimental sessions took place in School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. Participants visited the laboratory to complete questionnaires addressing health history and demographics, and to have height and weight assessed (*Seca Alpha, Hamburg, Germany*). Cardiorespiratory fitness ($\text{VO}_{2\text{MAX}}$) was measured using an incremental test to exhaustion on an electromagnetically braked cycle ergometer (*Lode Excalibur Sport, Groningen, Netherlands*). Following a three-minute warm up at 30 watts, workload was increased by 30 watts every minute, until volitional exhaustion. Oxygen uptake was assessed continuously using a breath-by-breath system (*Oxygon Prx, Jaeger, Wuerzburg, Germany*) and heart rate monitored using a Polar Vantage heart rate monitor (Polar Vantage, Kempele, Finland). The following criteria were used to indicate that $\text{VO}_{2\text{MAX}}$ had been reached: a fall in cadence below 60rpm, a respiratory exchange ratio (VCO_2/VO_2) >1.10 - 1.15 , plateau in participant oxygen consumption or a maximal heart

rate $>220 \text{ beats min}^{-1}$ - age (Howley et al., 1995). $\text{VO}_{2\text{MAX}}$ was expressed relative to body weight ($\text{ml.kg}^{-1}\text{min}^{-1}$).

5.3.3. Exercise Trials

Seven days after preliminary measurements, participants returned to the laboratory following an overnight fast to undertake one of three exercise trials, separated by at least seven days, in a randomised design. Prior to each exercise trial, subjects undertook a warm up (5 minutes) at a workload eliciting 40% $\text{VO}_{2\text{MAX}}$. Exercise trials were: two workload matched steady-state cycling trials at moderate (60% $\text{VO}_{2\text{MAX}}$; 27 minutes, MOD) and high (80% $\text{VO}_{2\text{MAX}}$; 20 minutes, HIGH) intensity and a low volume high intensity interval training (LV-HIIT) trial. LV-HIIT consisted of ten 1 minute cycling intervals at 90% $\text{VO}_{2\text{MAX}}$, with 1 minute low intensity cycling at 40% $\text{VO}_{2\text{MAX}}$ (figure 5.1).

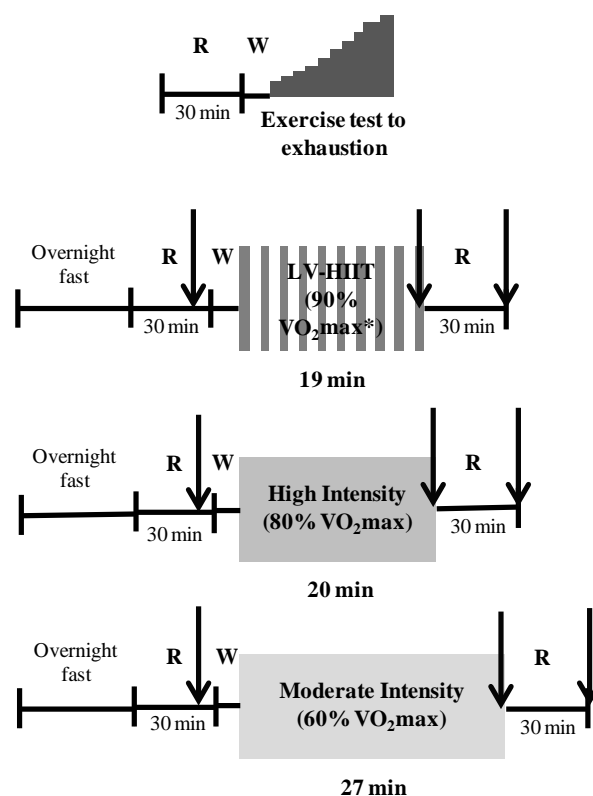


Figure 5.1: Experimental protocol. Subjects completed an exercise test to exhaustion and then participated in 3 subsequent exercise trials. LV-HIIT consisted of ten 1 minute stages at 90% $\text{VO}_{2\text{MAX}}$, interspersed with nine 1 minute intervals at 40% $\text{VO}_{2\text{MAX}}$.

↓ Blood samples (baseline, exercise & post+30); R, Rest; W, Warm up

5.3.4. Blood sampling

Prior to exercise, a catheter (*Becton, Dickson & Company, Oxford, UK*) was inserted into the antecubital vein of the arm and a rested blood sample drawn after thirty minutes of supine rest (baseline). The catheter was kept patent with saline (0.9% NaCl). Subsequent blood samples were taken during the last minute of exercise (exercise) and then 30 minutes following the exercise trial (post+30). At each time point, 20 ml of blood was drawn into four separate vacutainer tubes containing potassium ethylene diaminetetraacetic acid (EDTA) (*Becton, Dickson & Company, Oxford, UK*). Blood (5 ml) was then centrifuged at 2800 rpm for 15 minutes at 4 °C, plasma extracted and stored at -80 °C until further analyses.

5.3.5. Blood Cell Isolation

Three EDTA tubes (approximately 15 ml) from each time point were used to isolate PBMCs from whole blood using density gradient centrifugation. Briefly, whole blood was diluted 1:1 with Roswell Park Memorial Institute Media (RPMI), and then layered carefully on top of Ficoll paque PLUS (GE Healthcare) (2:1), before centrifuging at 1,350g for 30 minutes at 21°C. The PBMC layer was aspirated and then washed three times with RPMI, by centrifuging steps at 1600rpm for 5 minutes.. The final cell pellet was divided into two equal aliquots. The first aliquot was lysed using RIPA buffer (1x, *Sigma Aldrich*) containing a protease inhibitor cocktail (1µl/ml, *Sigma Aldrich*), vortexed thoroughly and lysate collected. The second aliquot was resuspended in a freezing mixture (RPMI, fetal calf serum (FCS) and

dimethyl sulfoxide (DMSO); 7:2:1) and frozen at -1°C /min using a freezing container (Nalgene “Mr Frosty” Thermoscientific). Both aliquots were stored at -80°C until further analyses.

5.3.6. Analytical Procedures

Whole blood cell counts (i.e., total leukocyte differential) were assessed using the coulter principle (*Coulter Analyser, Beckman-Coulter, High Wycombe, UK*). Haemoglobin (g/dL) and haematocrit (%) were assessed to calculate plasma volume changes as a result of exercise (Kargotich et al., 1997). Protein concentration was determined using the bicinchoninic assay method (Smith et al., 1985).

Validation of PRDX-SO₂₋₃ (I-IV) Antibody: oxidation of PRDX-1

The PRDX-SO₂₋₃ (I-IV) antibody was validated using a recombinant PRDX-1 protein (ab79945, *Abcam, Cambridge, UK*). PRDX was oxidised using peroxynitrite (Packer and Murphy, 1994). Solutions of hydrogen peroxide (0.6 M HCl and 0.7 M hydrogen peroxide) and sodium nitrite (0.6M) were thoroughly mixed (5 minutes) and added to rapidly stirring sodium hydroxide (2 M) to arrest the reaction. Four separate oxidising solutions were made (0, 35, 70, 140 mM peroxynitrite) and mixed 1:1 with PRDX-1 recombinant protein (1.25 μg). PRDX samples were incubated for one hour at 37°C . Western blotting was carried out as below to assess the amount of PRDX-1 and PRDX-SO₂₋₃ (I-IV).

Western Blotting Protocol

All reagent mixtures were sonicated thoroughly prior to use. Standards (PRDX-1 protein (peroxynitrite: 0-140 mM), 1.25 μg) and PBMC protein lysates (10 μg) were mixed 1:1 with laemmli sample buffer (10% 2-mercaptoethanol, *Sigma Aldrich, Dorset, UK*) and

separated on 15-18% polyacrylamide gels. Gels were electrophoresed at 115V for 105 minutes using electrophoresis buffer (25 mM Tris, 192 mM glycine, and 0.1% w/v SDS). Proteins were transferred onto Hybond-P® PVDF membrane (GE Healthcare, Amersham, UK) with transfer buffer (25 mM Tris, 192 mM glycine, and 20% w/v methanol) for 105 minutes at 170mA. Transfer was assessed by Ponceau S (*Sigma Aldrich, Dorset, UK*) before membranes were washed with sodium hydroxide (0.1 M) and then blocked overnight in non-fat milk (5%) in TBST blocking buffer (0.21 M NaCl, 0.05 M Tris Base, 0.1% w/v tween). Membranes were washed 6 times (5 minutes) in TBST (0.21 M NaCl, 0.05 M Tris Base, 0.05% w/v tween-20) prior to incubation with rabbit polyclonal for anti-PRDX-SO₂₋₃ (I-IV) (1:500, ab16830), rabbit polyclonal anti-PRDX-1 (1:1000, ab15571), mouse monoclonal anti-TRX-1 (1:1000, ab16965, *Abcam, Cambridge, UK*), and mouse monoclonal anti-actin (1:10,000, *Sigma Aldrich, Dorset, UK*) antibodies for 2 hours at room temperature. Membranes were washed (6x5 min) before peroxidase conjugated goat anti-rabbit (for PRDX and PRDX SO₂₋₃ (I-IV) work, 1:10,000, A6154) or goat anti-mouse (for TRX-1 and actin work, 1:10,000, A0168) antibodies (*Sigma Aldrich, Dorset, UK*) were applied for 1 hour at room temperature. Following a further washing step (6 x 5 min), visualisation of proteins was undertaken using Amersham ECL Prime detection reagent (*GE Healthcare, Amersham, UK*). Imaging and band quantification was assessed using Syngene G:Box F3 (*Geneflow, Staffordshire, UK*) and Syngene tools software respectively, and expressed in arbitrary units.

Thioredoxin Reductase Activity

PBMCs were rapidly thawed in a water bath (37°C). Pellets were washed twice in RPMI and FCS (9:1) to discard excess DMSO. Approximately 2 million cells were counted using a haemocytometer and aliquoted for each time point (baseline, exercise and post+30). All samples were adjusted to the lowest sample protein concentration. The lysate was then

assessed for TRX reductase enzyme activity using a commercially available kit according to manufacturer instructions (ab83463, *Abcam, Cambridge, UK*). Briefly, assay buffer (10 μ l) or TRX reductase inhibitor (10 μ l) were added to two sets of identical samples (50 μ l, diluted to 0.55mg/ml protein using assay buffer). All samples were then incubated with a reaction mix (30 μ l assay buffer + 8 μ l 5,5'-dithiobis (2-nitrobenzoic) acid (DNTB) + 2 μ l nicotinamide adenine dinucleotide phosphate (NADH) per well) and absorbances (λ = 412 nm) determined immediately and at 25 minutes to monitor reaction kinetics (Labsystems Multiskan MS, Virginia, *USA*). Values were then obtained from a linear standard curve of known 5-thio-2-nitrobenzoic acid (TNB) concentrations (0-50 nmol/well).

Interleukins

Plasma concentrations of IL-6 and IL-10 were determined using commercially available high-Sensitivity ELISA kits according to manufacturer instructions (*R&D Systems, Oxfordshire*). Briefly, samples (200 μ l, diluted 1:1 with assay diluent) were incubated with a monoclonal antibody (200 μ l) derived against IL-6, and then substrate (50 μ l) and amplifier (50 μ l) solutions added to develop the colour change. Plates were washed in-between stages to reduce non-specific binding. The reaction was stopped using 2 M sulphuric acid (50 μ l) and absorbance's read at 650 nm. Values were then obtained from a linear standard curve (0.156-10 pg/ml IL-6) and adjusted for changes in plasma volume (Kargotich et al., 1997).

To determine IL-10 concentrations, samples (250 μ l, diluted 4:1 with assay diluent) were incubated with a monoclonal antibody (200 μ l) derived against IL-10 and then substrate (50 μ l) and amplifier (50 μ l) solutions added to develop a colour change. Plates were washed intermittently to reduce non-specific binding. The reaction was stopped using 2 M sulphuric acid (50 μ l) and absorbance read at 490 nm. Values were then obtained from a linear standard curve of known IL-10 concentrations (0.78-50 pg/ml) and adjusted for plasma volume.

5.3.11. Statistical Analysis

Statistical analyses were performed using SPSS (PASW Statistics, release 21.0, SPSS Inc., Chicago, IL, USA). Differences between variables at baseline were assessed using one-way analyses of variance (ANOVA). The Kolmogorov Smirnov test was used to test for normally distributed data at all time points. Changes to the oxidation of recombinant PRDX protein (antibody validation) were assessed by paired samples t-tests. TRX-1 and PRDX-SO₃ protein expression changes in response to exercise were analysed using the Friedman's test, with Wilcoxon signed-ranked pairwise comparisons. Changes in PRDX-1 protein expression, TRX-R activity and inflammatory cytokines in response to exercise were assessed by repeated measures ANOVA. Post hoc analysis of any interaction effects was performed by a test of simple effects by pairwise comparisons, with Bonferroni correction. Statistical significance was accepted at the $p < .05$ level.

5.4. Results

5.4.1. Exercise Physiology Data

Table 5.1 reports the average workload and energy expenditure data following the three exercise trials. Energy expenditure following the LV-HIIT trial was significantly lower than both MOD ($p<0.0001$) and HIGH ($p<0.0001$) trials. There were no statistical differences in energy expenditure between MOD and HIGH.

	Average Workload (Watts)	Energy Expenditure (Kcal)	Difference in EE relative to LV-HIIT (%)
MOD	109.84 ± 17.89	$263.61 \pm 39.43^*$	27.75
HIGH	169.35 ± 31.50	$266.37 \pm 39.30^*$	28.50
LV-HIIT	210.60 ± 38.30	190.46 ± 29.83	-

Table 5.1: Exercise Physiology Data: Average workload (Watts), energy expenditure (Kcal) and percentage difference (%) in energy expenditure relative to LV-HIIT in the three exercise trials (n=10). * indicates a significant difference in energy expenditure, relative to LV-HIIT.

5.4.2. White Blood Cell Data

Table 5.2 reports the changes in lymphocyte number, monocyte number and lymphocyte: monocyte ratio in response to the three exercise trials. Lymphocyte number increased during exercise in all trials ($p<0.0001$) and returned to baseline post+30 ($p<.0001$). A significant time*condition interaction was observed ($p<.0001$) and pairwise comparisons revealed that the increase in lymphocyte number during HIGH was greater than both MOD ($p=0.002$) and LV-HIIT ($p=0.032$). Monocyte number increased during exercise in all trials

($p=0.010$) and returned to baseline levels post+30 ($p=.004$). No differences were observed in monocyte number between trials. No statistical differences were observed in lymphocyte: monocyte ratio in response to any of the exercise trials.

		Lymphocytes ($\times 10^6/\text{ml}$)	Monocytes ($\times 10^6/\text{ml}$)	Lymphocyte: Monocyte Ratio
MOD	Baseline	1.89 ± 0.47	0.29 ± 0.15	7.60 ± 3.04
	During	$2.67 \pm 0.75^*$	$0.43 \pm 0.21^*$	7.62 ± 4.91
	Post+30	1.67 ± 0.35	0.22 ± 0.08	8.57 ± 3.51
HIGH	Baseline	1.86 ± 0.42	0.32 ± 0.18	7.44 ± 3.58
	During	$4.63 \pm 1.41^{* \#}$	$0.60 \pm 0.40^*$	9.65 ± 5.06
	Post+30	1.78 ± 0.39	0.31 ± 0.14	6.76 ± 3.14
LV-HIIT	Baseline	1.79 ± 0.47	0.23 ± 0.11	10.02 ± 6.50
	During	$3.57 \pm 1.40^*$	$0.40 \pm 0.12^*$	10.27 ± 6.09
	Post+30	1.62 ± 0.38	0.33 ± 0.16	5.66 ± 1.98

Table 5.2: White Blood Cell Data: Lymphocyte, Monocyte and Lymphocyte: Monocyte Ratio data in response to exercise in the MOD, HIGH and LV-HIIT trials ($n=10$). * indicates a significant difference relative to baseline. # indicates a significantly greater response during HIGH relative to MOD and LV-HIIT.

5.4.3. Validation of antibody: Peroxynitrite Oxidation (35-140mM)

Figure 5.2 shows the changes in PRDX-1 protein expression and PRDX over-oxidation following oxidation using peroxynitrite (35-140 mM), expressed in arbitrary units.

Figure 5.3 illustrates two example western blotting images of PRDX-SO₂₋₃ (I-IV). PRDX-SO₂₋₃ (I-IV) was significantly greater following exposure with 140mM peroxynitrite, relative to 35mM ($p=.027$). Figure 5.4 illustrates a western blotting image of PRDX-1 protein expression following no manipulation, and oxidation using peroxynitrite (0-140mM). The PRDX-1 band was not detectable following oxidation with peroxynitrite, irrespective of concentration ($p<.0001$) (figure 5.2).

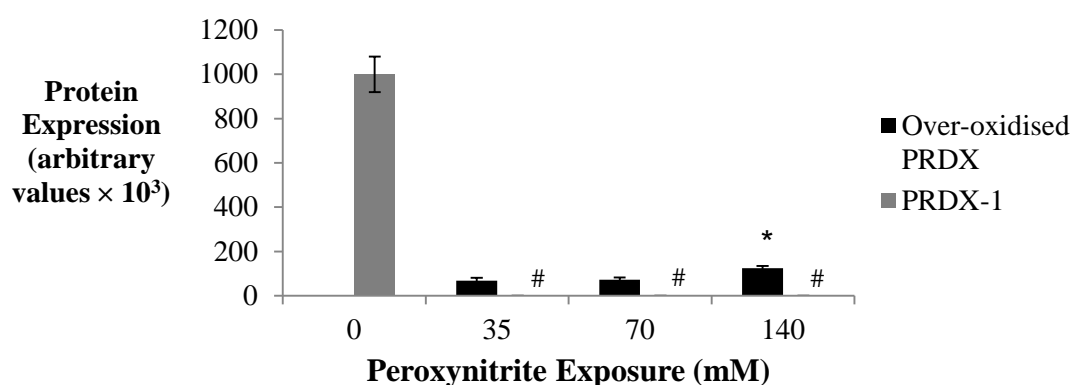


Figure 5.2: Changes in protein expression of PRDX-1 and PRDX-SO₂₋₃ in response to peroxynitrite exposure ($n=2$). * indicates significant difference in PRDX-SO₂₋₃ relative to 35mM peroxynitrite ($p<0.05$). # indicates significant difference in PRDX relative to 0mM peroxynitrite ($p<0.05$).

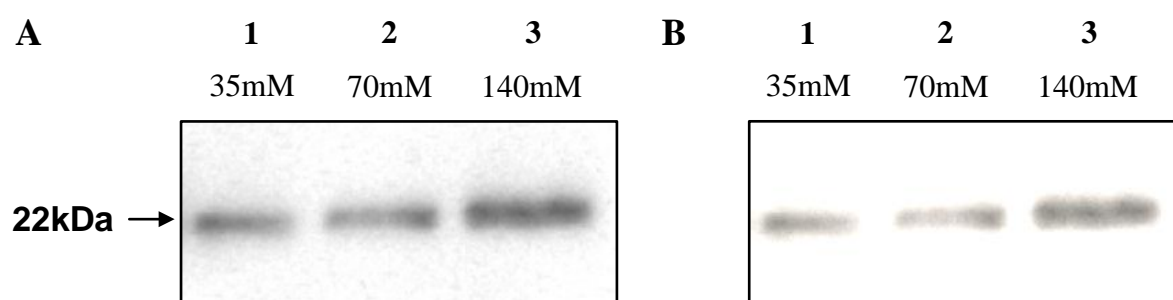


Figure 5.3: Two example western blots (A and B) for PRDX-SO₂₋₃ (I-IV) following PRDX-1 oxidation with peroxynitrite. Lane 1, PRDX-1 (1.25 μ g, 35 mM peroxynitrite); Lane 2, PRDX-1 (1.25 μ g, 70 mM peroxynitrite); Lane 3, PRDX-1 (1.25 μ g, 140 mM peroxynitrite).

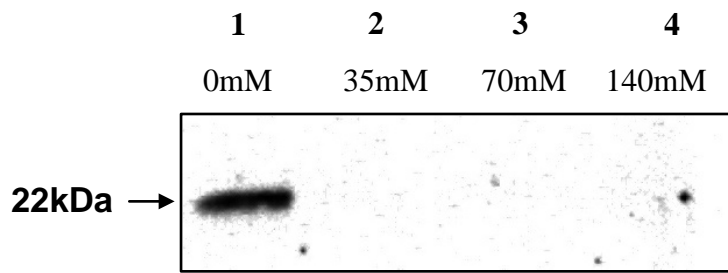


Figure 5.4: Western blot of PRDX-1 following PRDX-1 oxidation with peroxynitrite. Lane 1, PRDX-1 (1.25 μ g): Lane 2, PRDX-1 (1.25 μ g, 35 mM peroxynitrite); Lane 3, PRDX-1 (1.25 μ g, 70 mM peroxynitrite); Lane 4, PRDX-1 (1.25 μ g, 140 mM peroxynitrite).

5.4.4. Thioredoxin, Peroxiredoxin-1, Peroxiredoxin-SO₂₋₃ (I-IV) and actin protein expression changes

Figures 5.5 and 5.6 indicate changes in the amount of TRX-1 and PRDX SO₂₋₃ (I-IV) in the three exercise trials following quantification with Genetools (arbitrary values). TRX-1 increased in response to exercise in MOD ($X^2(2) = 8.600$, $p=0.014$), HIGH ($X^2(2) = 6.200$, $p=0.045$) and LV-HIIT ($X^2(2) = 11.436$, $p=0.003$). Post hoc analyses with Wilcoxon signed-rank tests revealed a significant increase in TRX-1 from baseline to during exercise in the LV-HIIT trial only ($Z = -2.666$, $p=0.008$). However, no differences were observed in TRX-1 protein expression during exercise between the three trials, i.e. increases in TRX-1 were not exercise intensity dependent. PRDX-1 was not detectable in any of the samples. PRDX-SO₂₋₃ increased in response to HIGH only ($X^2(2) = 7.824$, $p=0.020$). Post hoc analyses revealed that PRDX- SO₂₋₃ increased during HIGH, relative to baseline ($Z = -2.524$, $p=.012$). No statistical differences were observed in actin protein expression, relative to baseline in each trial or between trials.

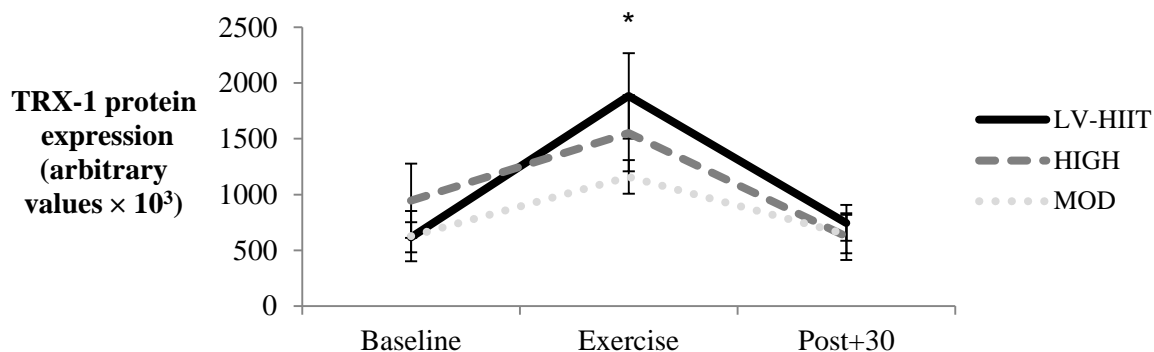


Figure 5.5: Total TRX-1 levels in response to the three exercise trials (n=10). Data points represent total TRX-1 in PBMC lysates before (Baseline), during (Exercise) and thirty minutes following exercise (Post+30). Values are means \pm standard error. * indicates significant differences during exercise, relative to baseline in all trials ($p < 0.05$).

MOD (.....), HIGH (---) and LV-HIIT (—).

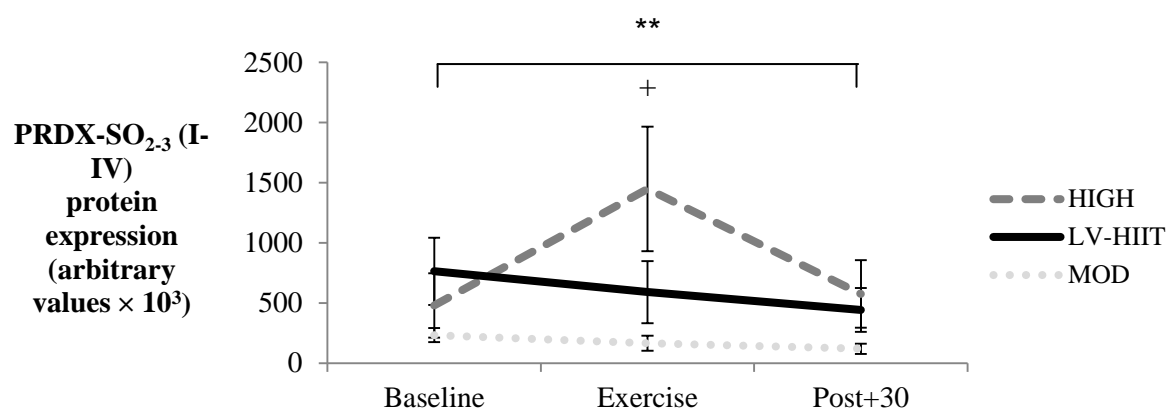


Figure 5.6: Total PRDX-SO_{2.3} (I-IV) levels in response to the three exercise trials (n=10). Data points represent total PRDX-SO_{2.3} (I-IV) in PBMC lysates before (Baseline), during (Exercise) and thirty minutes following exercise (Post+30). Values are means \pm standard

error. ** indicates differences between values in the different trials ($p < 0.05$). + indicates a significant difference between baseline and during exercise in HIGH ($p < 0.05$).

MOD (-----), HIGH (— — —) and LV-HIIT (————).

Figure 5.7 presents an example (1 participant) of the changes in TRX-1 (Fig 5.7A), PRDX-SO₂₋₃ (I-IV) (Fig 5.7B) and actin (Fig 5.7C) in response to the three exercise trials.

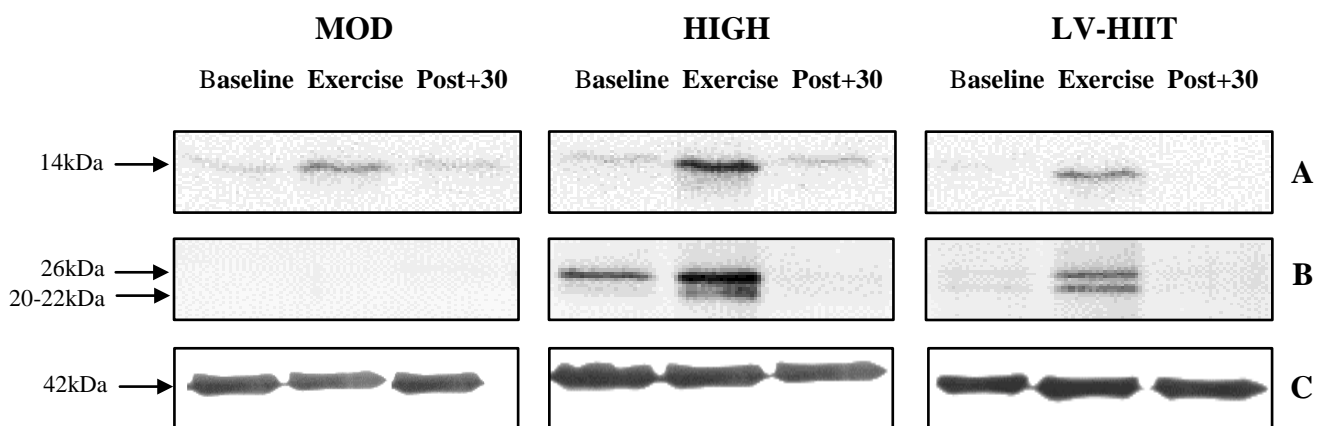


Figure 5.7: Changes in total TRX-1 (A), PRDX-SO₂₋₃ (I-IV) (B) and actin (C) protein content in participant 1 in response to the three exercise trials. The two distinct bands in figure 5.7B are representative of the different isoforms of PRDX. PRDX isoforms 1 and 2 resolve at 20-22kDa, PRDX-3 at 26kDa and PRDX-4 at 31kDa (no band).

5.4.5. Thioredoxin Reductase Activity

Figure 5.8 indicates changes in Thioredoxin reductase activity from baseline to during exercise in the three exercise trials. Three trial by two time ANOVAs revealed that TRX reductase activity increased during exercise, relative to baseline in all trials ($p = 0.002$). A significant group x time interaction effect was found ($p = 0.010$), and a test of simple effects analysis by pairwise comparisons indicated that TRX reductase enzyme activity during exercise was greater in HIGH than MOD ($p = 0.042$). Differences between HIGH and LV-HIIT did not reach statistical significance ($p = 0.123$).

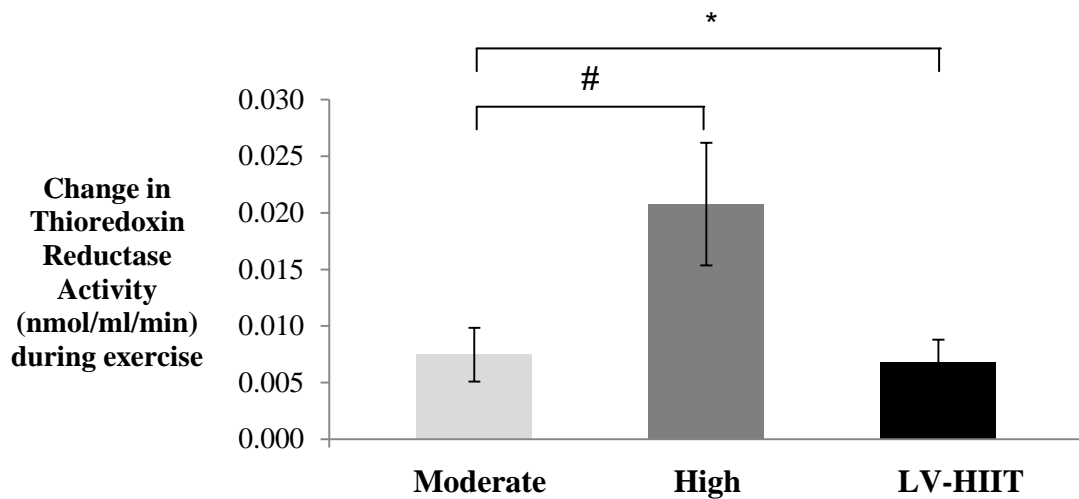


Figure 5.8: Changes in Thioredoxin Reductase Activity in response to the three exercise trials during exercise (n=10). Filled bars represent changes in Thioredoxin Reductase Activity in PBMC lysates from baseline to during exercise. Values are means \pm standard error. * indicates significant changes in response to exercise, relative to baseline in all trials ($p<0.05$). # indicates a significant difference between HIGH and MOD during exercise ($p<0.05$).

5.4.6. Interleukins

Figures 5.9 (A-B) indicates changes in IL-6 (Fig 5.9A) and IL-10 (Fig 5.9B) in response to the three exercise trials, as assessed by three trial by three time ANOVAs. These results are reported in a previous paper from our group (Wadley et al, 2014). IL-6 increased during exercise ($p=0.05$) and post+30 ($p=0.001$) in all trials. A significant group \times time interaction effect was found ($p=0.012$), and a test of simple effects analysis by pairwise comparisons indicated that the concentrations of IL-6 post+30 were significantly higher in HIGH than the MOD ($p=0.037$). There were increases in IL-6 during exercise (LV-HIIT: $p=0.029$; HIGH: $p=0.048$) and post+30 (LV-HIIT: $p=0.010$; HIGH: $p=0.016$) relative to baseline in the LV-HIIT and HIGH. IL-6 increased post+30, relative to baseline in MOD

($p=0.10$). There were no statistical differences between the responses observed in LV-HIIT and HIGH.

IL-10 was unchanged during exercise, however a significant increase was observed post+30 in HIGH and LV-HIIT trials (main effect of time: $p=0.042$). A significant group \times time interaction effect was found ($p=0.015$), and IL-10 post+30 was greater in HIGH than MOD ($p=0.05$). IL-10 was increased post+30 relative to baseline ($p=0.05$) and during exercise ($p=0.05$) in LV-HIIT and HIGH respectively. There were no statistical differences in the IL-10 responses observed during the LV-HIIT and HIGH.

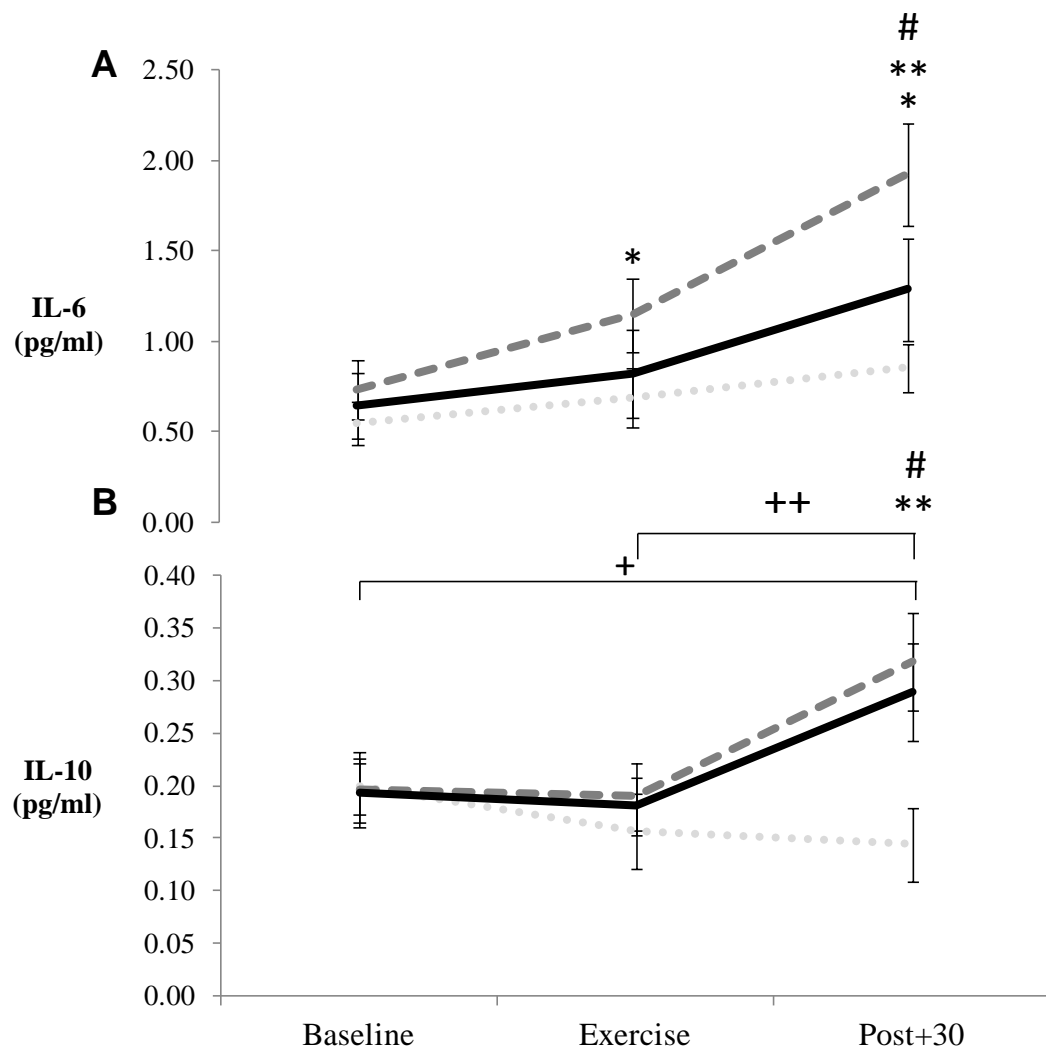


Figure 5.9: Changes in IL-6 (A) and IL-10 (B) in response to the three exercise trials. Values are means \pm standard error. * indicates significant differences relative to baseline in all trials

($p < .05$). ** indicates significant differences relative to during exercise in all trials. # indicates a significant difference between HIGH and MOD. + indicates a significant difference between baseline and post+30 in LV-HIIT. ++ indicates a significant difference between exercise and post+30 in HIGH.

MOD (.....), HIGH (— — —) and LV-HIIT (————).

5.4.7. Correlation Analysis

A significant positive correlation was observed between the changes in PRDX-SO₂₋₃ (I-IV) and plasma IL-6 from baseline to during exercise in HIGH ($r = .561$, $p = .046$). No correlation was observed between changes in PRDX-SO₂₋₃ (I-IV) and IL-10 from baseline to during exercise in HIGH ($r = .266$, $p = .229$).

5.5. Discussion

This study demonstrates that PBMC TRX-1 protein content increased during exercise in young healthy males, irrespective of exercise intensity. An increase in over-oxidation of PRDX isoforms (I-IV) was shown during exercise in HIGH only. The activity of TRX-R, the key regulatory enzyme of both redox cycles, increased during exercise in all trials, with the greatest response observed in HIGH. In line with previous work linking redox proteins with inflammatory cytokine production, systemic IL-6 correlated with the increases in PRDX over-oxidation in HIGH.

To our knowledge this is the first study assessing changes in PBMC TRX-1 protein content in response to single bouts of exercise in humans. Previously, TRX-1 protein expression in PBMCs of mice increased following 30 minutes of swimming exercise (Sumida et al., 2004). In response to heightened oxidative stress, cytosolic TRX-1 is released in a RONS-dependent manner from its binding protein Thioredoxin-interacting protein (TXNIP) (Zhou et al., 2010) to elicit an array of antioxidant actions. These include directly scavenge RONS (Kang, 1998), reducing oxidised macromolecules (Burke-Gaffney et al., 2005) and regulating the expression/recycling of antioxidant proteins such as manganese superoxide dismutase (Das, 2004) and reduced glutathione (Tan et al., 2010). The results of the current study and our previous work that demonstrated an increase in plasma markers of oxidative stress (e.g., lipid hydroperoxides and total antioxidant capacity) during all three exercise trials (Wadley et al., 2013), indicate a presence of both intra- and extra-cellular RONS respectively. It appears that exercise-induced RONS may have stimulated the release of TRX-1 from TXNIP (i.e. cleavage of disulphide bond), via TRX-R in all trials. Thus, we suggest that exercise has stimulated an antioxidant response in PBMCs during exercise.

Increases in PRDX over-oxidation in PBMCs during exercise likely occurs in response to higher levels of RONS such as hydrogen peroxide and peroxynitrite (Silveira et al., 2003). The catalytic cysteine residue of PRDX can utilise its peroxidase activity to regulate peroxide mediated cellular signalling and toxicity within lipid rafts (Wood et al., 2003) (Rhee et al., 2012). In the current study, a significant increase in over-oxidation of PRDX was observed during exercise in HIGH, suggesting that peroxide exposure was highest in this trial and/or that TRX reductase activity was sufficient in the other trials to limit PRDX peroxidase activity. In addition, lower exercise-induced peroxide exposure in MOD and LV-HIIT may have limited PRDX cysteine oxidation to the initial oxidation state, sulfenic acid. In this conformational change, a PRDX cysteine residue forms a disulphide bond with an adjacent PRDX molecule (Rhee et al., 2005). A threshold may therefore exist, whereby exercise-induced RONS exposure may exceed TRX-1 regulatory control and over-oxidise PRDX. This threshold may have been exceeded during exercise in HIGH. Given that PRDX-1 was not detectable in any of the samples in the current study (using a PRDX-1 antibody) and over-oxidised PRDX-4 was also not detectable, we suggest that only PRDX isoforms II and III were over-oxidised during HIGH. However, it is clear that recombinant PRDX-1 is extremely susceptible to peroxynitrite exposure (Figure 5.4) and it must be acknowledged that the purchased PRDX-1 antibody may have weak affinity for PRDX-1 in isolated human cells.

PRDX isoforms (I-IV) are known to be predominantly regulated by the TRX redox cycle (Peskin et al., 2007; Rhee et al., 2005), in particular the NADH dependent enzyme TRX-R. TRX-R activity increased during all exercise trials, with the greatest increase observed in HIGH. This too suggests an antioxidant response to exercise as the enzyme attempts to reform reduced PRDX in NADH dependent manner.

Both TRX (Maurice et al., 1999) and PRDX (Shichita et al., 2012) have been previously associated with heightened inflammation. Indeed, in the current study a significant

correlation was observed between changes in PRDX-SO₂₋₃(I-IV) and changes in IL-6 from baseline to during exercise in HIGH. Recent evidence has demonstrated that following TRX-TXNIP dissociation, TXNIP can directly induce increases in IL-1 β , an early inflammatory cascade cytokine (Zhou et al., 2010) and signal for IL-6 production (Lorre et al., 1994). Similarly, PRDX has been linked with IL-23 (Shichita et al., 2012) and IL-6 production in macrophages (Riddell et al., 2010) Low PRDX content in lymphocytes is associated with uncontrolled immune activation that may lead to subsequent cytokine production (Lorre et al., 1994; Michalek et al., 2012). Conversely, high PRDX content in pro-inflammatory macrophages, that are derived from monocytes have also been associated with cytokine production (Boytard et al., 2013). Recent evidence has suggested that PRDX mediated cytokine production in pro-inflammatory macrophages is independent of the peroxidase activity of PRDX, but instead is related to chaperone activity (Riddell et al., 2010). This study does not seek to present a mechanism for the observed association between PRDX over-oxidation and cytokine production. It is however clear that further research is necessary to understand the interactions and relationships between the redox cycle of PBMCs and the inflammatory response/immune cell function.

It must be noted that despite normalisation for total cell and protein content, shifts in the number and phenotype of lymphocytes and monocytes can occur during exercise (Tauler et al., 2006; Campbell et al., 2009). Indeed, lymphocyte and monocyte number did increase in response to exercise in all trials (table 5.2). However, previous work has indicated that shifts in the cellular composition of peripheral blood do not influence biomarkers of oxidative stress (Turner et al., 2011). Finally, a limitation to the current study is that PRDX (II-III) protein expression was not measured to quantify the relative oxidised to reduced ratio of PRDX proteins. Despite no significant differences in baseline PRDX-SO₂₋₃(I-IV), figure 5.7B clearly indicates baseline variation in the example participant. Recent evidence has

indicated that peroxiredoxin oxidation displays a circadian rhythm (Edgar et al, 2012) and although all exercise trials were performed at the same time of day, this data indicates how basal metabolism can influence redox homeostasis.

In conclusion, the present results show that both TRX-1 and PRDX are perturbed in response to exercise in PBMC's of untrained males. Whilst similar increases in TRX-1 were observed in response to all exercise trials, only HIGH caused over-oxidation of PRDX which appeared to exceed the regulatory control of TRX-R. In addition, PRDX over-oxidation during HIGH was associated with increases in systemic IL-6. Although the implications of PRDX over-oxidation in response to exercise are currently unknown, these data support links between oxidative stress (redox protein status changes) and inflammation in response to exercise.

5.6. Reference list for chapter five

Aldred, S. (2007) Oxidative and nitrative changes seen in lipoproteins following exercise. **Atherosclerosis**, 192 (1): 1–8

Boytard, L., Spear, R., Chinetti-Gbaguidi, G., et al. (2013) Role of proinflammatory CD68(+) mannose receptor(-) macrophages in peroxiredoxin-1 expression and in abdominal aortic aneurysms in humans. **Arteriosclerosis, thrombosis, and vascular biology**, 33 (2): 431–8

Brinkmann, C. and Brixius, K. (2013) Peroxiredoxins and sports: new insights on the antioxidative defense. **The journal of physiological sciences : JPS**, 63 (1): 1–5

Burke-Gaffney, A., Callister, M.E. and Nakamura, H. (2005) Thioredoxin: friend or foe in human disease? **Trends Pharmacol Sci**, 26 (8): 398–404

Campbell, J.P., Riddell, N.E., Burns, V.E., et al. (2009) Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. **Brain, behavior, and immunity**, 23 (6): 767–75

Das, D.K. (2004) Thioredoxin regulation of ischemic preconditioning. **Antioxidant Redox Signal**, 6: 405–412

Edgar, R.S., Green, E.W., Zhao, Y. (2012) Peroxiredoxins are conserved markers of circadian rhythms. **Nature**, 485: 459–464

Gomez-Cabrera, M.C., Domenech, E. and Vina, J. (2008) Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. **Free Radic Biol Med**, 44 (2): 126–131

Holmgren, A. and Lu, J. (2010) Thioredoxin and thioredoxin reductase: current research with special reference to human disease. **Biochemical and biophysical research communications**, 396 (1): 120–4

Howley, E.T., Bassett Jr, D.R. and Welch, H.G. (1995) Criteria for maximal oxygen uptake: a review and commentary. **Medicine & Science in Sports & Exercise**, 27 (9): 1292–1301

Kang, S.W. (1998) Characterization of a Mammalian Peroxiredoxin That Contains One Conserved Cysteine. **Journal of Biological Chemistry**, 273 (11): 6303–6311

Kargotich, S., Goodman, C., Keast, D., et al. (1997) The influence of exercise-induced plasma volume changes on the interpretation of biochemical parameters used for monitoring exercise, training and sport. **Sports medicine**, 7 (3): 185–191

- Lorre, K., Karan, A., Van Vaeck, F., et al. (1994) Interleukin-1 and B7/CD28 interaction regulate interleukin-6 production by human T cells. **Clinical Immunology and Immunopathology**, 70 (1): 81–90
- Low, F., Hampton, M., and Winterbourn, C. (2008) Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. **Antioxidant Redox Signal**, 10: 1621–1630
- Marumoto, M., Suzuki, S., Hosono, A., et al. (2010) Changes in thioredoxin concentrations: an observation in an ultra-marathon race. **Environ Health Prev Med**, 15 (3): 129–134
- Maurice, M.M., Nakamura, H., Gringhuis, S., et al. (1999) Expression of the thioredoxin-thioredoxin reductase system in the inflamed joints of patients with rheumatoid arthritis. **Arthritis Rheum**, 42 (11): 2430–2439
- Messina, J.P. and Lawrence, D.A. (1989) Cell cycle progression of glutathione-depleted human peripheral blood mononuclear cells is inhibited at S phase. **Journal of immunology (Baltimore, Md. : 1950)**, 143 (6): 1974–81
- Michalek, R.D., Crump, K.E., Weant, A.E., et al. (2012) Peroxiredoxin II regulates effector and secondary memory CD8+ T cell responses. **Journal of virology**, 86 (24): 13629–41
- Packer, M.A. and Murphy, M.P. (1994) Peroxynitrite causes calcium efflux from mitochondria which is prevented by Cyclosporin A. **FEBS letters**, 345 (2-3): 237–40
- Peskin, A. V., Low, F.M., Paton, L.N., et al. (2007) The high reactivity of peroxiredoxin 2 with H₂O₂ is not reflected in its reaction with other oxidants and thiol reagents. **The Journal of biological chemistry**, 282 (16): 11885–92
- Radak, Z., Chung, H.Y. and Goto, S. (2005) Exercise and hormesis: oxidative stress-related adaptation for successful aging. **Biogerontology**, 6 (1): 71–5
- Rhee, S.G., Chae, H.Z. and Kim, K. (2005) Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. **Free radical biology & medicine**, 38 (12): 1543–52
- Rhee, S.G., Jeong, W., Chang, T.S., et al. (2007) Sulfiredoxin, the cysteine sulfinic acid reductase specific to 2-Cys peroxiredoxin: its discovery, mechanism of action, and biological significance. **Kidney Int Suppl.**, 106: S3–8
- Rhee, S.G., Woo, H.A., Kil, I.S., et al. (2012) Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides. **The Journal of biological chemistry**, 287 (7): 4403–10

Riddell, J.R., Wang, X.Y., Minderman, H., et al. (2010) Peroxiredoxin 1 stimulates secretion of proinflammatory cytokines by binding to TLR4. **Journal of immunology (Baltimore, Md. : 1950)**, 184 (2): 1022–30

Schenk, H., Klein, M., Erdbrügger, W., et al. (1994) Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. **Proc Natl Acad Sci U S A**, 91 (5): 1672–1676

Schenk, H., Vogt, M., Droge, W., et al. (1996) Thioredoxin as a potent costimulus of cytokine expression. **J Immunol**, 156 (2): 765–771

Shichita, T., Hasegawa, E., Kimura, A., et al. (2012) Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. **Nature medicine**, 18 (6): 911–7

Silveira, L.R., Lucia Pereira-Da-Silva, C.J. and Hellsten, Y. (2003) Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. **Free Radical Biology and Medicine**, 35 (5): 455–464

Smith, P.K., Krohn, R.I., Hermanson, G.T., et al. (1985) Measurement of protein using bicinchoninic acid. **Analytical Biochemistry**, 150 (1): 76–85

Spindel, O.N., World, C. and Berk, B. (2011) Thioredoxin Interacting Protein (TXNIP): Redox dependent and independent regulatory mechanisms. **Antioxid Redox Signal**

Sumida, S., Nakamura, H. and Yodoi, J. (2004) Thioredoxin Induction of Peripheral Blood Mononuclear Cells in Mice in Response to a Single Bout of Swimming Exercise. **Gen. Physiol. Biophys.**, 23: 241–249

Sureda, A., Tauler, P., Aguiló, A., et al. (2005) Relation between oxidative stress markers and antioxidant endogenous defences during exhaustive exercise. **Free radical research**, 39 (12): 1317–24

Szabó-Taylor, K.É., Eggleton, P., Turner, C.A.L., et al. (2012) Lymphocytes from rheumatoid arthritis patients have elevated levels of intracellular peroxiredoxin 2, and a greater frequency of cells with exofacial peroxiredoxin 2, compared with healthy human lymphocytes. **The international journal of biochemistry & cell biology**, 44 (8): 1223–31

Tan, S.X.X., Greetham, D., Raeth, S., et al. (2010) The thioredoxin-thioredoxin reductase system can function in vivo as an alternative system to reduce oxidized glutathione in *Saccharomyces cerevisiae*. **Journal of Biological Chemistry**, 285 (9): 6118–6126

Tauler, P., Sureda, A., Cases, N., et al. (2006) Increased lymphocyte antioxidant defences in response to exhaustive exercise do not prevent oxidative damage. **The Journal of nutritional biochemistry**, 17 (10): 665–71

Turner, J.E., Bennett, S., Campbell, J., et al. (2013) The antioxidant enzyme peroxiredoxin-2 is depleted in lymphocytes seven days after ultra-endurance exercise. **Free radical research**, 47 (10): 821–8

Turner, J.E., Bosch, J.A., Drayson, M.T., et al. (2011) Assessment of oxidative stress in lymphocytes with exercise. **Journal of applied physiology (Bethesda, Md. : 1985)**, 111 (1): 206–11

Wadley, A.J., Chen, Y.W., Lip, G.Y., et al. (2013) Effect of low volume high intensity interval and steady state exercise bouts on oxidative stress and inflammation in humans. **Med Sci Sports Exerc**, (under review)

Wood, Z.A., Poole, L.B. and Karplus, P.A. (2003) Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. **Science (New York, N.Y.)**, 300 (5619): 650–3

Zhou, R., Tardivel, A., Thorens, B., et al. (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. **Nat Immunol**, 11 (2): 136–140

Chapter Six

6. General Discussion

6.1 Summary

The work undertaken in this thesis has investigated how markers of oxidative stress and inflammation respond to exercise. A primary focus was to explore some of the factors which might alter these responses. For example: chapters 4 and 5 investigated the impact of exercise intensity on the oxidative stress and inflammatory responses to acute exercise; and chapters 2 and 3 aimed to assess the impact of altered baseline inflammation on the oxidative stress response to acute exercise, exercise training and psychological stress. An overarching aim of this thesis was to identify associations between oxidative stress and inflammation, in response to exercise. Commonly assessed markers of oxidative stress (lipid peroxidation, protein oxidation and total antioxidant capacity) were measured throughout this thesis to allow comparisons between the responses of different participant groups used in the studies presented.

6.2 Influence of exercise intensity on oxidative stress

Chapters 4 and 5 investigated markers of oxidative stress and inflammation in response to exercise of different intensities. Many researchers agree that the increases observed in oxidative stress in response to exercise are not directly related to total workload (St-Pierre et al., 2002; Quindry et al., 2003; Di Meo and Venditti, 2001), but that exercise intensity (Bailey et al., 2004) and duration (Bloomer et al., 2007) might be more influential factors. Data presented in chapters 4 and 5 collectively support the hypothesis and previous studies (Berzosa et al., 2011; Wang and Huang, 2005), indicating that changes in selective markers of oxidative stress in plasma and PBMC are exercise intensity dependent. Changes in mean plasma lipid oxidation were different between bouts (LOOH (nmol/ml plasma): moderate +0.36; high +3.09; LV-HIIT +5.51) although the difference in magnitude of

response did not reach statistical significance. Notably, changes in TAC were not intensity dependent, which supports previous work (Berzosa et al., 2011). Chapter 5 further explored redox specific changes in PBMCs in response to these bouts. PRDX over-oxidation was intensity dependent when comparing the steady state bouts, but not in LV-HIIT, supporting a relationship previously shown when assessing lipid oxidation in PBMCs (Wang and Huang, 2005). PRDX may therefore utilise its antioxidant capacity to limit peroxide mediated lipid oxidation at higher steady state exercise intensities in PBMCs (Rhee et al., 2012). Conversely, TRX-1 might act to limit lipid oxidation during LV-HIIT, given that TRX-1 was elevated to the greatest degree in this bout (MOD +84.5%; HIGH +64.1%; LV-HIIT +205.7%; $p > .05$), without a concurrent rise in PRDX oxidation.

Findings from chapters 4 and 5, together with supporting literature suggest that lipid oxidation is enhanced in response to higher exercise intensities in both plasma and PBMCs. This is likely to reflect increased structural remodelling of cell membranes, which might protect cells against future exercise bouts. Whereas changes in the oxidation state of PRDX may act to limit lipid oxidation at higher steady state exercise intensities, it appears that TRX may regulate this during LV-HIIT. This suggests that the regulation of exercise-induced RONS may differ depending on the modality of the exercise. This has wider implications for beginning to explain the surprisingly comparable adaptations that LV-HIIT conveys relative to steady state exercise (Rognmo et al., 2004; Wisløff et al., 2007; Hood et al., 2011). However, given that no direct measures of lipid oxidation or PRDX protein expression were made within PBMCs in chapter 5, this remains speculative.

These studies also investigated the response of factors that influence inflammation. Results support previous findings that plasma IL-6 and IL-10 display an intensity dependent response to steady state exercise (Ostrowski et al., 2000; Fischer, 2006), and adds new data characterising the response to LV-HIIT. The magnitude of change in IL-6 and IL-10 in

response to LV-HIIT was more comparable to high, rather than moderate intensity steady state exercise. Given that LV-HIIT provided a far lower physiological insult (demonstrated by the cardiovascular and adrenaline responses) than high intensity steady state exercise, these data may advocate the use of LV-HIIT as an anti-inflammatory exercise modality in a variety of populations.

In beginning to understand how markers of oxidative stress can be differentially altered at higher exercise intensities, it is important to consider how the stimulus (i.e. radical source) may ultimately form a specific adduct. Evidence from chapter two provides support, showing how two inflammatory stimuli can alter different markers of oxidative stress.

6.3. The impact of inflammation on oxidative stress

6.3.1. Acute Inflammation

The first experimental study in this thesis employed two well characterised laboratory models of inducing inflammation, namely vaccination and eccentric exercise, to monitor the impact of altered baseline inflammation on the oxidative stress response to mental stress. The results showed that vaccination and eccentric exercise increased markers of inflammation and selective plasma markers of oxidative stress prior to the start of a mental stress task. Despite a significant cardiovascular and immune response to mental stress, markers of oxidative stress were unchanged in response to mental stress, regardless of the observed inflammatory status. Within the time frame examined post-stress in this study, acute changes in systemic oxidative stress appear to provide no insight into the mechanisms behind mental stress induced MI.

Using experimental models in healthy individuals in this way showed that two different inflammatory signals (muscle damage and mild pathogen) both induced transient

increases in IL-6, but that the inflammatory stimulus altered different markers of plasma oxidative stress. Links between inflammation and oxidative stress are well accepted (Wadley et al., 2012; Csiszar et al., 2009; Ungvari et al., 2010), although the precise signalling mechanisms are not completely clear. Eccentric exercise induces muscle tissue damage that is followed by an influx of migrating phagocytic cells from the circulation which can release RONS to facilitate the breakdown of damaged muscle fibres (Pincemail et al., 1990). Conversely, vaccination introduces a mild pathogen to the body that the immune system acts to dispose of by mobilising and activating phagocytic cells, such as granulocytes (table 2.2) that can release RONS (Babior et al., 1973). The results show that vaccination stimulates significant changes in systemic protein oxidation and nitric oxide metabolism, whereas eccentric exercise elicits a significant antioxidant response. Interestingly, mean TAC concentrations increased and decreased in response to eccentric exercise and vaccination respectively. Differences may be explained by respiratory bursts from different white blood cell subsets. Indeed, shifts in peripheral blood composition were different between inflammatory conditions (tables 2.2 and 2.4), with a more pronounced rise in phagocytic cells in the circulation 6 hours following vaccination. Nevertheless, these data underpin the unique aspect of oxidative events following specific acute inflammatory signalling.

6.3.2. Acute and Chronic Inflammation

The second study in this thesis (chapter three) aimed to establish the impact of acute and chronic exercise on markers of oxidative stress in patients with RA, a condition that is associated with increased systemic and localised inflammation (Seven et al., 2008; Taysi et al., 2002; Sarban et al., 2005). In assessing oxidative stress perturbations in this population,

this allowed a comparison of disease associated chronic baseline inflammation with acute manipulations of baseline inflammation (chapter two).

Plasma protein oxidation and metabolites of nitric oxide significantly increased in response to a single bout of exercise (heart rate eliciting 70% $\text{VO}_{2\text{MAX}}$). Following three months of regular exercise training at the same intensity, patients fitness and disease activity score improved (assessed by $\text{VO}_{2\text{MAX}}$ and DAS28) and there was a reduction in plasma free and bound 3-NT. Additional systemic markers of oxidative stress (PC, LOOH, TAC and CAT) and inflammation (IL-8 and CRP) were unchanged following exercise training. Evidence from this chapter refutes the hypothesis that exercise would stimulate reductions in basal oxidative stress over a period of aerobic exercise training. However, due to baseline inflammation and oxidative stress being low in these participants, this result was not so surprising.

This study provides the first evidence that the response of oxidative stress to exercise in RA appears similar to the responses seen in healthy populations (Turner et al., 2011a; Bloomer et al., 2007; Aldred and Rohalu, 2011). Despite acute increases in selective markers of oxidative stress (i.e. protein carbonyls) following moderate intensity exercise (30-40 minutes, 70% $\text{VO}_{2\text{MAX}}$), over a period of training, basal level of oxidative stress and inflammation were not increased. In addition, when drawing comparisons of the markers employed in chapter 4 and previous work in our laboratory (Turner et al., 2011a), the acute oxidative stress response to exercise was not exaggerated in RA. Supporting literature has suggested that aerobic exercise of an intensity exceeding 85% HR_{MAX} in patients with heart disease and chronic obstructive pulmonary disorder (COPD) can simulate increases in systemic oxidative stress that exceed those of healthy populations (Avogaro et al., 1986; Nishiyama et al., 1998; Van Helvoort et al., 2006). When considering the implications of these studies for exercise guidelines in RA, it is possible that exercising at higher intensities

may elicit changes in markers of oxidative stress and inflammation that surpass the increases observed in age-matched controls. Indeed, it must be noted that some studies have suggested that oxidation is greater within the exercising joint of an RA patient, compared to a control subject (Zhang et al, 1993). The pathophysiology of heart disease and COPD may differ significantly from RA and therefore direct comparisons are difficult to make. Importantly, it still remains far from clear the exact biological significance of the exaggerated responses observed in these populations.

It must be noted that the ‘typical’ antioxidant response noted in response to exercise in chapters 2, 4 and 5 and supporting studies (Turner et al., 2011a; Berzosa et al., 2011) was not observed following moderate intensity exercise in RA patients. Given that resting TAC concentrations were lower in RA patients compared to the healthy participants in chapters 2 and 4 (RA: 819.0 ± 121.1 vs. Health range: $935.2 - 1260.4 \mu\text{M}$), it might be speculated that an antioxidant response to exercise was prevented by a compromised antioxidant status at baseline. Given the accrued stress placed on the body with exercise of a continuous higher intensity nature (chapter 4), LV-HIIT may provide an attractive alternative in RA. However, considering the intensity dependent relationship of oxidative stress with exercise discussed previously, reduced antioxidant protection might be expected to exacerbate exercise-induced oxidative stress with any higher intensity exercise.

6.4. Low volume high intensity interval training as tool in health and disease

LV-HIIT is known to initiate a diverse range of metabolic, vascular and physiological benefits that parallel adaptations observed following conventional steady state exercise in a range of populations (Rognmo et al., 2004; Wisløff et al., 2009). Results in chapters 4 and 5 add to the growing body of support by reporting that a single bout of LV-HIIT can elicit anti-

inflammatory and anti-oxidant effects during and following exercise. Strikingly, at a significantly reduced energy expenditure (\approx -30%), cardiovascular (\approx -32%) and adrenaline (\approx -38%) response compared to high intensity steady state exercise, LV-HIIT can stimulate comparable increases in IL-6, IL-10, TAC and TRX in response to exercise in young and healthy individuals. In the context of human disease, despite the documented health benefits of LV-HIIT in low-grade inflammatory diseases (Little et al., 2012; Wisløff et al., 2009), it remains unclear as to its effects on oxidative stress, inflammation and general health parameters in high grade inflammatory diseases, such as RA.

6.5. Hormesis

The recently proposed theory of exercise induced oxidative stress and hormesis suggests that there is a theoretical threshold whereby high levels of exercise-induced RONS may prevent adaptation, potentially compromising immunity and initiating tissue damage (Radak et al., 2005). Certain factors such as health status and the type of exercise (i.e. intensity) may exacerbate the exercise-induced RONS response and exceed this threshold. Data in this thesis provides no evidence to support the hormesis threshold concept. Results in chapter 3 indicate that exercise in RA causes transient increases in oxidative stress, however repeated bouts do not exacerbate basal oxidative stress. Exercise training in RA was also associated with improvements in disease activity and aerobic fitness, therefore suggesting that exercise was not harmful. Furthermore, results in chapters 4 and 5 indicate that transient increases in oxidative stress during exercise, even at high intensities, return to baseline within 30 minutes. It must be noted that some studies do provide support for the hormesis threshold (Turner et al., 2013, 2011b). However, although these studies observed a depleted antioxidant

status up to 28 days following ultra-endurance exercise, this mode of exercise, at least in the general population, is far from conventional.

6.6. Conclusions

Increases in oxidative stress are well established in skeletal muscle (Khassaf et al., 2003; Petersen et al., 2012; Khassaf et al., 2001), plasma (Goldfarb et al., 2002; Bloomer et al., 2005; Turner et al., 2011b) and PBMCs (Sureda et al., 2005; Tauler et al., 2006; Turner et al., 2011a) in response to exercise in humans. The research in this thesis supports evidence that exercise can cause changes in systemic makers of oxidative stress both during and following steady state, interval-based and eccentric exercise. The work presented here adds to this evidence base with the following novel findings:

1. A mental stress task did not stimulate acute increases in oxidative stress, irrespective of experimentally manipulated high baseline inflammation. This suggests that increased systemic oxidative stress is not the mechanism behind mental stress induced MI.
2. Acute increases in oxidative stress following moderate intensity exercise in RA are transient. Basal levels of oxidative stress and inflammation were not increased by a course of exercise training in a group of 'disease stable' RA patients.
3. The plasma oxidative stress response to a single bout of LV-HIIT did not significantly differ to steady state exercise.
4. A bout of LV-HIIT resulted in a lower energy expenditure, cardiovascular stress (i.e. heart rate and blood pressure) and plasma adrenaline response, but elicited a comparable anti-inflammatory and anti-oxidant response to high intensity steady state exercise.

5. TRX increased in peripheral blood mononuclear cells during exercise. The magnitude of change was greatest during the LV-HIIT bout.
6. PRDX oxidation increased in peripheral blood mononuclear cells during high intensity steady state exercise. PRDX oxidation was associated with increases in plasma IL-6, adding support for the links between PRDX and inflammation, but in the context of exercise.

6.7. Reference list for chapter six

- Aldred, S. and Rohalu, M. (2011) A moderate intensity exercise program did not increase the oxidative stress in older adults. **Arch Gerontol Geriatr**, 53 (3): 350–353
- Avogaro, P., Bon, G.B., Cazzolato, G., et al. (1986) Plasma Malondialdehyde in Coronary Patients and in “ Normal ” People at Rest and After Exercise. **Cardiovascular Medicine and Surgery**, 13: 291–296
- Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. **Journal of Clinical Investigation**, 52 (3): 741–744
- Bailey, D.M., Young, I.S., McEneny, J., et al. (2004) Regulation of free radical outflow from an isolated muscle bed in exercising humans. **American journal of physiology. Heart and circulatory physiology**, 287 (4): H1689–99
- Berzosa, C., Cebrian, I., Fuentes-Broto, L., et al. (2011) Acute exercise increases plasma total antioxidant status and antioxidant enzyme activities in untrained men. **J Biomed Biotechnol**, pp. 1–7
- Bloomer, R.J., Davis, P.G., Consitt, L.A., et al. (2007) Plasma protein carbonyl response to increasing exercise duration in aerobically trained men and women. **International Journal of Sports Medicine**, 28 (1): 21–25
- Bloomer, R.J., Goldfarb, A.H., Wideman, L., et al. (2005) Effects of acute aerobic and anaerobic exercise on blood markers of oxidative stress. **Journal of strength and conditioning research / National Strength & Conditioning Association**, 19 (2): 276–85
- Csiszar, A., Labinskyy, N., Jimenez, R., et al. (2009) Anti-oxidative and anti-inflammatory vasoprotective effects of caloric restriction in aging: role of circulating factors and SIRT1. **Mech Ageing Dev**, 130 (8): 518–527
- Fischer, C.P. (2006) Interleukin-6 in acute exercise and training: what is the biological relevance? **Exercise immunology review**, 12 (115): 6–33
- Goldfarb, A.H., You, T., Bloomer, R.J., et al. (2002) Blood oxidative stress markers in response to aerobic exercise: effect of gender. **Med Sci Sports Exerc**, 34: S429
- Van Helvoort, H.A.C., Heijdra, Y.F., Thijs, H.M.H., et al. (2006) Exercise-induced systemic effects in muscle-wasted patients with COPD. **Medicine and science in sports and exercise**, 38 (9): 1543–52

Hood, M.S., Little, J.P., Tarnopolsky, M.A., et al. (2011) Low-volume interval training improves muscle oxidative capacity in sedentary adults. **Medicine and science in sports and exercise**, 43 (10): 1849–56

Khassaf, M., Child, R.B., McArdle, A., et al. (2001) Time course of responses of human skeletal muscle to oxidative stress induced by nondamaging exercise. **J Appl Physiol**, 90 (3): 1031–1035

Khassaf, M., McArdle, A., Esanu, C., et al. (2003) Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. **Journal of Physiology**, 549 (2): 645–652

Little, J.P., Gillen, J.B., Percival, M.E., et al. (2012) Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes. **Journal of Applied Physiology**, 111: 1554–1560

Di Meo, S. and Venditti, P. (2001) Mitochondria in exercise-induced oxidative stress. **Biol Signals Recpt.**, 10 (1-2): 125–140

Nishiyama, Y., Ikeda, H., Haramaki, N., et al. (1998) Oxidative stress is related to exercise intolerance in patients with heart failure. **American Heart Journal**, 135: 115–120

Ostrowski, K., Schjerling, P. and Pedersen, B.K. (2000) Physical activity and plasma interleukin-6 in humans effect of intensity of exercise. **European journal of applied physiology**, 83 (6): 512–5

Petersen, A.C., McKenna, M.J., Medved, I., et al. (2012) Infusion with the antioxidant N-acetylcysteine attenuates early adaptive responses to exercise in human skeletal muscle. **Acta physiologica (Oxford, England)**, 204 (3): 382–92

Pincemail, J., Camus, G., Roesgen, A., et al. (1990) Exercise induces pentane production and neutrophil activation in humans. Effect of propranolol. **European Journal of Applied Physiology and Occupational Physiology**, 61 (3-4): 319–322

Quindry, J.C., Stone, W.L., King, J., et al. (2003) The effects of acute exercise on neutrophils and plasma oxidative stress. **Med Sci Sports Exerc**, 35 (7): 1139–1145

Radak, Z., Chung, H.Y. and Goto, S. (2005) Exercise and hormesis: oxidative stress-related adaptation for successful aging. **Biogerontology**, 6 (1): 71–5

Rhee, S.G., Woo, H.A., Kil, I.S., et al. (2012) Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides. **The Journal of biological chemistry**, 287 (7): 4403–10

Rognmo, Ø., Hetland, E., Helgerud, J., et al. (2004) High intensity aerobic interval exercise is superior to moderate intensity exercise for increasing aerobic capacity in patients with coronary artery disease. **European Journal of Cardiovascular Prevention & Rehabilitation**, 11: 216–222

Sarban, S., Kocyigit, A., Yazar, M., et al. (2005) Plasma total antioxidant capacity, lipid peroxidation, and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. **Clinical biochemistry**, 38 (11): 981–6

Seven, A., Guzel, S., Aslan, M., et al. (2008) Lipid, protein, DNA oxidation and antioxidant status in rheumatoid arthritis. **Clinical Biochemistry**, 41 (7-8): 538–543

St-Pierre, J., Buckingham, J.A., Roebuck, S.J., et al. (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. **The Journal of biological chemistry**, 277 (47): 44784–90

Sureda, A., Tauler, P., Aguiló, A., et al. (2005) Relation between oxidative stress markers and antioxidant endogenous defences during exhaustive exercise. **Free radical research**, 39 (12): 1317–24

Tauler, P., Sureda, A., Cases, N., et al. (2006) Increased lymphocyte antioxidant defences in response to exhaustive exercise do not prevent oxidative damage. **The Journal of nutritional biochemistry**, 17 (10): 665–71

Taysi, S., Polat, F., Gul, M., et al. (2002) Lipid peroxidation, some extracellular antioxidants, and antioxidant enzymes in serum of patients with rheumatoid arthritis. **Rheumatol Int**, 21 (5): 200–204

Turner, J.E., Bennett, S., Campbell, J., et al. (2013) The antioxidant enzyme peroxiredoxin-2 is depleted in lymphocytes seven days after ultra-endurance exercise. **Free radical research**, 47 (10): 821–8

Turner, J.E., Bosch, J.A., Drayson, M.T., et al. (2011a) Assessment of oxidative stress in lymphocytes with exercise. **Journal of applied physiology (Bethesda, Md. : 1985)**, 111 (1): 206–11

Turner, J.E., Hodges, N.J., Bosch, J.A., et al. (2011b) Prolonged Depletion of Antioxidant Capacity after Ultraendurance Exercise. **Medicine & Science in Sports & Exercise**, 43 (9): 1770–1776

Ungvari, Z., Kaley, G., de Cabo, R., et al. (2010) Mechanisms of vascular aging: new perspectives. **J Gerontol A Biol Sci Med Sci**, 65 (10): 1028–1041

Wadley, A.J., Veldhuijzen van Zanten, J.J.C.S. and Aldred, S. (2012) The interactions of oxidative stress and inflammation with vascular dysfunction in ageing : the vascular health triad. **AGE**, 35 (3): 705–718

Wang, J.-S. and Huang, Y.-H. (2005) Effects of exercise intensity on lymphocyte apoptosis induced by oxidative stress in men. **European journal of applied physiology**, 95 (4): 290–7

Wisløff, U., Ellingsen, K. and Kemi, O.J. (2009) High-Intensity Interval Training to Maximize Cardiac Benefits of Exercise Training ? **Exercise and sport sciences reviews**, pp. 139–146

Wisløff, U., Støylen, A., Loennechen, J.P., et al. (2007) Superior Cardiovascular Effect of Aerobic Interval Training Versus Moderate Continuous Training in Heart. **Circulation**, 115: 3086–3094

Zhang, Z., Farrel, A.J., Blake, D.R., Chidwick, K. and Winyard, P.G. (1993) Inactivation of synovial fluid alpha 1-antitrypsin by exercise of the inflamed rheumatoid joint. **Clinical Molecular and Microbiology Papers**, 321 (2-3): 247-278