THE DEVELOPMENT OF A FLUORESCENT DUAL LABELLING TECHNIQUE FOR THE QUANTITATIVE MEASUREMENT OF REDUCED AND OXIDISED PROTEIN THIOLS IN BIOLOGICAL TISSUE, AND ITS APPLICATION TO EXERCISE AND RECOVERY IN MUSCLES OF RATS AND HUMANS

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This thesis is submitted as partial requirement for the Doctor of Philosophy at the University of Western Australia

June 2012
EXECUTIVE SUMMARY

Reactive oxygen and nitrogen species (RONS) are produced by tissues during normal resting metabolism. One major site of RONS production is skeletal muscle, with its rate of RONS production increasing during exercise. There is evidence that the resulting rise in RONS concentrations plays many important physiological roles. One important mechanism through which RONS operate involves the reversible oxidation of exposed protein thiols of the amino acid cysteine. Unfortunately, as reviewed in chapter one, there are many pitfalls with the protein thiol assays currently available, and research into this field has been hampered by a lack of suitable analytical techniques. For this reason, the primary aim of the first study of this thesis (chapter two) is to develop a technique to measure protein thiols in biological tissues in both their reduced and oxidised states.

Chapter two describes a highly sensitive, quantitative labelling technique that measures the level of global and specific protein thiol oxidation in skeletal muscle. The technique involves the labelling of the reduced and oxidised protein thiols within the same complex tissue sample with different fluorescent dyes. The resulting sample is assayed using a 96-well plate fluorimeter, and individual protein bands are separated using SDS PAGE and later identified using mass spectrometry. We show that artefactual oxidation during sample preparation and analysis has the potential to confound results, and techniques to prevent this are described. We tested the technique by analysing the muscles of mdx (dystrophic) and c57 mice, and found the muscles of mdx mice were significantly (p < 0.05) more oxidised (13.1 ± 1.5 % oxidised thiols) than those of c57 mice (8.9 ± 0.7 % oxidised thiols). This technique provides an effective means to
measure the extent to which oxidative stress affects the oxidation of protein thiols in biological tissues.

Using the dual labelling technique developed in chapter two, the primary aim of chapter three was to examine the effect of both three-minutes high intensity swimming to fatigue and 30 minutes of moderate intensity non-fatiguing swimming on the level of muscle protein thiol oxidation in overnight fasted male Wistar rats. We showed that the response of protein thiol oxidation to exercise is muscle fibre specific. Indeed, in response to both moderate intensity swimming and high intensity swimming to fatigue, there was a significant (p < 0.05) increase in the level of thiol oxidation of total proteins in the red \textit{gastrocnemius}. In contrast, these two exercise protocols had no effect on the level of protein thiol oxidation of total proteins in the white \textit{gastrocnemius} muscle, and resulted in a fall in the level of protein thiol oxidation of total proteins in the \textit{soleus} muscle (p < 0.05). Both exercise protocols had no effect on protein carbonyl levels in the muscles examined. During the recovery period following high intensity exercise, the level of protein thiol oxidation in the red \textit{gastrocnemius} muscle increased transiently, peaking at 15 minutes before slowly declining over the following 45 minutes of recovery. Furthermore, exercise and recovery resulted in changes in the level of protein thiol oxidation that were specific in both magnitude, and in some instances directional change, to target proteins. In this respect, myosin, glycogen phosphorylase and troponin appear to be proteins sensitive to thiol oxidation in response to exercise induced RONS production. On the basis of these findings it was concluded that the changes in the level of protein thiol oxidation occur in an exercise, fibre and protein-specific manner.
The aforementioned findings in rats raise the question of whether high intensity exercise to fatigue also affects the level of thiol oxidation of muscle proteins in humans. Since this issue has never been addressed before, the primary aim of chapter four was to examine whether a maximal sprint to fatigue results in an increase in the level of thiol oxidation of muscle proteins and whether it returns progressively back towards pre-exercise levels during recovery. Following a familiarisation session, five healthy male athletes aged between 18 and 35 years old were required to perform a 30-second maximal sprint effort on a cycle ergometer following an overnight fast. Blood samples, muscle oxygenation level, and muscle biopsies from the vastus lateralis were performed before exercise and at 0, 15 and 40 minutes into the recovery period. In response to sprinting, there was a 40% fall in muscle power together with a 47% decrease in muscle oxygenation. Labelling of reduced and oxidised protein thiols revealed a 37% decrease in the level of thiol oxidation of total proteins. 1D SDS PAGE was used to quantify the level of thiol oxidation of major protein bands. Following the maximal sprint, there was a 39% decrease in myosin oxidation, a 7.3% decrease in glycogen phosphorylase and no significant change in actin. During the 40 minutes recovery period, the level of thiol oxidation of total muscle proteins as well as muscle oxygenation level increased and remained elevated. On the basis of these findings, it is concluded that sprinting causes a transient decrease in the level of thiol oxidation of total muscle proteins followed by a post-exercise increase in the level of protein thiol oxidation above resting levels. We propose that these patterns of changes during and after exercise may be involved in muscle fatigue and muscle adaption to training, respectively.
DECLARATION

I, Alex E. Armstrong, declare that this thesis is comprised solely of my own research and that the work included herein has not been previously submitted at any other university or institution. The work involved in designing and conducting the studies described in this thesis has been carried out primarily by Alex E. Armstrong (the candidate). The thesis outline and experimental design of the studies were developed and planned by the candidate in consultation with Professors Paul Fournier and A/Professor Peter Arthur (the candidate’s supervisors). The two supervisors have also provided feedback for further drafts and completion of the thesis. Ralf Zerbes helped with the lab work for the experiment described in Fig. 2.5, Amber Boyatzis helped with the mass spectrometry analyses described in Chapter three, and Anish Singh performed the muscle biopsies described in Chapter four.

Alex E. Armstrong  Professor Paul A. Fournier
B.Econ., B.Sc. (Hons)  Co-ordinating Supervisor
I would like to and thank the following people, who have made this PhD possible.

**To my supervisors:**

**Professor Paul Fournier.** Thank you for your insight, direction and help with co-ordinating these studies. Without your input this project would have not been possible.

**Associate Professor Peter Arthur.** Thank you for your time and tireless dedication to this project. Your knowledge of biochemistry, ability to interpret data and problem solve in the lab has been invaluable.

**To Mum and Dad:**

Thank you for your love, support and encouragement. You both provide me with great inspiration for academic success. Thank you for all the opportunities you have provided me that has enabled me to complete this PhD.

**To my partner Amanda:**

Thank you for you love and support. I am very grateful to have someone who can relate to the trials and tribulations of research and academic work.

You are an inspiration to me.

**To those who assisted me during this project:**

I would like to thank Ralf Zerbes for his assistance with the lab work for the experiments described in Fig. 2.5, Amber Boyatzis for her help with the mass spectrometry in Chapter three, and Anish Singh for performing the muscle biopsies in Chapter four.
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LIST OF ABBREVIATIONS

a.u. arbitrary units
ACN acetonitrile
AMPK AMP-activated protein kinase
ANOVA analysis of variance
APS ammonium persulfate
ATP adenosine triphosphate
ADP adenosine diphosphate
BSA bovine serum albumin
Ca$^{2+}$ Calcium
CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CPLA2 Calcium-dependent phospholipase A2
DNA deoxyribonucleic acid
DTNB 5,5'-dithiobis-(2-nitrobenzoic acid)
DNPH 2,4-dinitrophenylhydrazine
DTT dithiothrietol
EDTA ethylenediaminetetraacetic acid
Em emission
eNOS Nitric oxide synthase (endothelial form)
Ex excitation
Fe$^{3+}$ iron III
FLm FL-N-(2-aminoethyl) maleimide
GSSG glutathione disulphide
GSH  glutathione
H+  Hydrogen
HCl  Hydrochloric acid
kDa  kilodalton
M   Molar
Mdx  dystrophic mouse
mM  millimolar
mg  milligram
ml  millilitre
NaOH  sodium hydroxide
NAC  N-acetylcysteine
nmol  nanomolar
nNOS  nitric oxide synthase (neural form)
NEM  N-ethyl maleimide
NADPH  Nicotinamide adenine dinucleotide phosphate
NHMRC  National Health and Medical Research Council of Australia.
NIRS  near infra-red spectroscopy
Nm  nanometers
NO  nitric oxide
O2  oxygen
O2−  super oxide radical
ONO0  peroxinitrite
PCA  perchloric acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RONS</td>
<td>reactive oxygen and nitrogen species</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>sodium dodecyl sulfate poly acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH</td>
<td>sulfhydryl</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SSA</td>
<td>sulfosalicylic acid</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TRm</td>
<td>texas red maleimide</td>
</tr>
<tr>
<td>XOD</td>
<td>xanthine oxidase</td>
</tr>
<tr>
<td>UWA</td>
<td>University of Western Australia</td>
</tr>
<tr>
<td>VO₂max</td>
<td>Maximal volume of oxygen consumption</td>
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CHAPTER 1

LITERATURE REVIEW
1.1 THE EFFECT OF EXERCISE ON RONS PRODUCTION

Oxygen plays an important role in releasing the energy required to support a wide range of body functions including locomotion. In doing so, reactive oxygen species and reactive nitrogen species (RONS) are generated. RONS are chemically reactive molecules such as peroxynitrite, superoxide, hydroxyl radical and hydrogen peroxide (Halliwell and Chirico 1993). They are produced in a number of biological tissues in response to normal oxidative metabolism (Jackson et al. 1985) and their rate of production increases in response to stressors such as exercise (Jackson et al. 1985), ageing (Stadtman 2004), pollution (Halliwell 1991), injury (Van Der Meulen et al. 1997) and disease (Murphy and Kehrer 1989; Rando et al. 1998; Anderson et al. 2009). The production of RONS is opposed by a series of anti-oxidant systems which help maintain RONS concentrations within physiological limits. However, when RONS production exceeds anti-oxidant capacity, a state of oxidative/nitrosative stress develops. The term “oxidative stress” was originally defined by Sies and Cadenas as “a disturbance in the pro-oxidant/anti-oxidant balance in favour of the former” (Sies and Cadenas 1985). However, because of the complexity of cellular redox balance, this was redefined by Jones to “a disruption of redox signaling and control” (Jones 2006). More recently, Jones and Sies collaborated to further refine this definition to “an imbalance between oxidants and anti-oxidants in favour of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” (Sies and Jones 2007; Powers et al. 2011). This definition takes into account RONS ability to cause both molecular damage as well as alter cellular function through redox signaling and control.
Because RONS can be generated as a consequence of pathological processes (Sen 1995; Rabinovich et al. 2001; Dalle-Donne et al. 2006; Anderson et al. 2009), RONS were originally thought to be purely toxic molecules. This view was supported by the observations that high RONS concentrations can cause irreversible oxidative damage to cellular macromolecules such as DNA, membrane lipids and proteins. However, it is now clear that RONS play vital roles in many important cellular functions as discussed in detail in the following sections.

Skeletal muscles are among the many organs with the capacity to produce RONS (Kozlov et al. 2005). RONS production by skeletal muscle was shown to increase during muscular contraction almost thirty years ago (Davies et al. 1982; Jackson et al. 1985), an issue that has been extensively reviewed over the past decades (Sen 1995; Cassarino and others 1997; Reid 2001; Jackson 2007; Ferreira and Reid 2008; Powers and Jackson 2008; Fisher-Wellman and Bloomer 2009; Powers et al. 2011). The primary RONS generated by skeletal muscles both during rest and contraction are nitric oxide and superoxide, the latter of which dismutates rapidly to form hydrogen peroxide (Quintanilha and Packer 1983; McArdle and Jackson 2000; Jackson 2007). These molecules provide the precursors for the generation of other RONS such as peroxynitrite and hydroxyl radical (Patwell et al. 2004; Close et al. 2005; Jackson et al. 2007). This increased exercise-mediated production of RONS has been shown to be associated with an increase in glutathione oxidation and depletion (Sen et al. 1992), lipid peroxidation (Sumida et al. 1989; Duthie et al. 1990) and protein carbonylation (Barreiro et al. 2005), all of which are generally considered as markers of oxidative stress.
Although it is well established that both aerobic and anaerobic exercise can increase RONS production (Davies et al. 1982; Ashton et al. 1998; Ashton et al. 1999; Groussard et al. 2003; Fisher-Wellman and Bloomer 2009), with exercise-induced oxidative stress being dependent upon exercise intensity and duration (Goto et al. 2007; Fisher-Wellman and Bloomer 2009; Munoz Marin et al. 2010), the identity of the source of RONS production during muscle contraction remains to be firmly established.

1.2 SOURCE OF RONS IN SKELETAL MUSCLE

1.2.1 Mitochondrial electron transport chain

The intramuscular source of RONS production during contraction has been an area of much debate, with a number of metabolic processes likely to be involved (Jackson 2007). For several years, the mitochondrial production of superoxide from the incomplete reduction of oxygen from complexes I and III in the electron transport chain was proposed to be a primary source of RONS generation (Boveris and Chance 1973; Halliwell and Gutteridge 1989; Barja 1999; Muller et al. 2004). This view was consistent with both the observation that oxygen flux through skeletal muscle can increase by a factor of 100 during contraction and that superoxide production is related to increased respiratory chain activity during physical exercise (Halliwell and Gutteridge 1989; Sen 1995). However, this view was subsequently challenged on the basis that, during aerobic contraction, mitochondrial respiration moves from state four (basal) to state three (maximal ADP stimulated respiration), with mitochondrial production of RONS being lower in state three than in state four. This has led to the view that the rate of mitochondrial production of RONS during contraction is likely limited (Loschen et al. 1971; Herrero and Barja 1997; Di Meo and
Indeed, there is evidence that the upper limit of mitochondrial superoxide production is 0.15% of electron flow through the mitochondria, which is far less than the original estimate (Echtay et al. 2002; St-Pierre et al. 2002; Jackson et al. 2007). These findings are also consistent with the observation that intracellular RONS generation by contracting skeletal muscle increases by two to four fold during contraction (McArdle et al. 2005; Vasilaki et al. 2006; Jackson 2007), and not by a factor of 50-100 as was originally predicted (Sen 1995).

1.2.2 NADPH oxidase

NADPH oxidase is another potential source of RONS production in skeletal muscle during exercise. There are two different subtypes of NADPH oxidase; one is associated with the plasma membrane and the other with the sarcoplasmic reticulum (SR). Both isozymes are similar in that they both contain electron transfer components and regulatory subunits. In the presence of ADP and Fe$^{3+}$, NADPH oxidase catalyses one electron transfer from NADPH to oxygen, which produces superoxide radical (O$_2^\cdot$) (Ji 1999).

\[
O_2^+ + NADPH \rightarrow O_2^\cdot + NADP^+ + H^+
\]

NADPH oxidase associated with the plasma membrane releases O$_2^\cdot$ into the extracellular space. The SR-bound NADPH oxidase activity, on the other hand, is closely coupled to the activation of calcium (Ca$^{2+}$) release from the SR during contraction (Finaud et al. 2006), with its activity being pO$_2$ dependent (Sun et al. 2011). What still remains without an answer is the relative contribution of NADPH oxidase to RONS production during exercise in skeletal muscle.
1.2.3 Calcium-dependent phospholipase A2

Calcium-dependent phospholipase A2 (CPLA2) is another potential source of RONS production during exercise. This is supported indirectly by the observation that conditions that elevate cytoplasmic Ca\(^{2+}\) levels, such as muscle contraction, are also associated with increased RONS production (Gong et al. 2006). Under these conditions, the increased Ca\(^{2+}\) levels have the capacity to activate CPLA2 which stimulates RONS production. In this respect, CPLA2 has been reported to mediate the increase in intracellular RONS during repetitive muscle contraction and to be dependent on influx of extracellular Ca\(^{2+}\) via L type channel (Reid 2001). This implies that CPLA2 has the capacity to stimulate increased RONS production during aerobic exercise (Nethery et al. 1999). However, its relative importance as an exercise-mediated source of RONS production remains to be established.

1.2.4 Xanthine oxidase

Xanthine oxidase is another possible source of RONS production by skeletal muscles. During exercise at intensities above maximum oxygen uptake, tissue hypoxia can result as oxygen supply cannot match oxygen demand. Furthermore, blood flow and oxygen supply are markedly reduced during sustained muscular contractions that increase intramuscular pressure (Barnes 1980; Sjogaard et al. 1986). In both cases, the resulting ischemia is followed by an increase in muscle perfusion post-exercise which has the capacity to trigger the conversion of xanthine dehydrogenase to xanthine oxidase. The increase in the activity of xanthine oxidase causes the degradation of hypoxanthine to xanthine and results in the production of RONS including superoxide and hydrogen peroxide (Vina et al. 2000). As a result, xanthine oxidase activity, as well as RONS concentrations, increase after exercise (Hellsten et al. 1996; Volek et al. 2002). The importance of this mechanism of RONS
production is supported by studies in both animals and humans where free radical markers of oxidative stress are reduced when allopurinol (a xanthine oxidase inhibitor) is administered (Barclay and Hansel 1991; Vina et al. 2000; Gomez-Cabrera et al. 2003). However, one problem with this proposed mechanism, is that RONS generated via xanthine oxidase peaks post-exercise, thus indicating that the time frame for xanthine oxidase activation does not coincide with that of RONS production during exercise. Therefore, xanthine oxidase may be a more important mechanism of RONS production post-exercise.

It should be noted that while the xanthine oxidase mechanism of RONS production has been implicated in both rats and humans, rat skeletal muscle appears to contain significantly more xanthine oxidase than human skeletal muscle (Hellsten et al. 1996; Judge and Dodd 2004), so care must be taken when interpreting results from animal research and comparing it with humans. Further research is required to establish the extent that this enzyme has on RONS production in humans.

1.2.5 NO synthase

The production of nitric oxide (NO) and peroxynitrite (ONOO) depends on the activation of NO synthase. Of the different NO synthase isoforms found in skeletal muscles, there is evidence that the neural form, nNOS, is responsible for the increase in intramuscular NO production during physical activity (McConnell and Kingwell, 2006). Given that AMP-activated protein kinase (AMPK) catalyses the phosphorylation-mediated activation of NOS and that it is necessary for calcium-calmodulin to bind to nNOS before this enzyme can be activated, this provides a mechanism whereby changes in both AMP and calcium levels during exercise may mediate increased NO production (McConell and Kingwell
2006). Elevated NO levels together with increased superoxide levels would in turn be expected to result in a rise in ONOO levels. How important these mechanisms of RNS production are relative to total RONS production in mediating the oxidative-nitrosative stress associated with exercise remains to be determined.

1.2.6 **Other mechanisms of RONS production during exercise**

Other possible mechanisms of intramuscular RONS production have been proposed including auto-oxidation of myoglobin. However, the significance of these mechanisms in RONS production in working skeletal muscle remains to be determined (Reid 2001).

1.2.7 **Insufficient antioxidant defense during exercise**

Under normal physiological conditions, skeletal muscles have a number of anti-oxidant systems which remove RONS and maintain their concentrations within physiological ranges. These include cytosolic and mitochondrial superoxide dismutase, catalase, glutathione peroxidase, glutaredoxin and a number of non-enzymic scavengers such as glutathione, vitamin E and ascorbate (Jackson 2007; Palomero and Jackson 2010).

Of all the anti-oxidants, glutathione is the most abundant, existing in millimolar concentrations and far exceeding the concentrations of other anti-oxidants of the cell (Meister and Anderson 1983). GSH serves multiple functions in protecting tissues from RONS and is the main anti-oxidant responsible for maintaining the reduced state of the intra-cellular environment (Meister and Anderson 1983; Ji et al. 1993; Ferreira et al. 2008).
Since RONS concentrations reflect the balance between RONS generation and RONS removal, it follows that insufficient RONS removal rate may in theory contribute to the RONS accumulation during exercise. Under conditions where the increased RONS production during muscle contraction is not completely controlled by anti-oxidant systems, an increase in RONS concentrations occurs, leading to a state of “oxidative-nitrosative stress”.

1.3 RONS EFFECTS ON MUSCLE FUNCTION

1.3.1 RONS as a mediator of muscle adaptive response to exercise and disuse

RONS have been implicated in a variety of muscle functions including the adaptation of muscle to both physical activity and disuse. In fact, almost three decades ago, Davies and colleagues suggested that RONS produced by exercising muscles may contribute to muscle adaptation to physical activity by stimulating mitochondrial biogenesis (Davies et al. 1982; Reid 2001; Powers et al. 2011). Since then, others have reported that repeated bouts of exercise-induced oxidative stress leads to an up-regulation of the body’s anti-oxidant defense systems (Vincent et al. 2000; Gomez-Cabrera et al. 2008) as well as a shift in the cellular redox balance toward a more reduced environment, therefore providing an element of protection from subsequent bouts of exercise-induced oxidative stress (Elosua et al. 2003; Fatouros et al. 2004; Fisher-Wellman and Bloomer 2009; Powers et al. 2010). Furthermore, increased RONS production has been associated with the increased expression of several genes (McClung et al. 2010; Powers et al. 2010) including those involved in cell hypertrophy (Xie et al. 1999), the Na\(^+\)-K\(^+\) pump (Murphy et al. 2008), and glycogen metabolism to list a few (Silveira et al. 2006). Of interest, contraction-mediated
RONS production has been associated with the up-regulation of transcription factors such as NF-kB which in turn have the capacity to regulate the expression of several genes (Kabe et al. 2005; Kramer and Goodyear 2007).

The notion that RONS is a mediator of adaptive changes to exercise is based in part on the many studies which have shown that muscle gene expression in response to an acute bout of physical activity or exercise training is impaired in both humans and animals treated with antioxidants such as vitamin C, N-acetylcysteine (NAC) or other inhibitors of RONS production such as allopurinol (Gomez-Cabrera et al. 2005; Gomez-Cabrera et al. 2008; Murphy et al. 2008; Ristow et al. 2009; Ristow et al. 2009; Petersen et al. 2012). It should be noted, however, that others have reported otherwise (Yfanti et al. 2010; Higashida et al. 2011).

Paradoxically, nearly two decades ago, Kondo and colleagues provided evidence that RONS also play a role in the regulation of muscle atrophy associated with prolonged muscle disuse. They reported not only that immobilisation of skeletal muscles is associated with a rise in RONS production, but also that muscle atrophy is delayed by exogenous anti-oxidants (Kondo et al. 1992; Kondo et al. 1993; Kondo et al. 1993; Kondo 2000). More recently, these early observations have been confirmed by others (McClung et al. 2007). The observation that increase RONS concentrations mediate muscle responses to both acute and repeated physical activity and muscle disuse would appear to be contradictory. Although this apparent paradox appears to be without a definitive answer, it has been proposed that it is the duration and magnitude of the RONS response that may explain the
differences in RONS-mediated adaptation to exercise and muscle disuse (Powers et al. 2005; Powers et al. 2007).

1.3.2 **RONS as a mediator of optimum muscle contractility**

There is evidence from the work of Reid and colleagues with isolated muscle bundles that RONS may have a biphasic effect on muscle force. They found that while higher concentrations of RONS inhibit force production during fatiguing muscle contraction (Reid et al. 1992), in non-fatigued muscle, higher RONS concentrations actually improved force production, whereas lower RONS concentrations decreased force production (Reid et al. 1993). This suggests that RONS are essential for optimal contractility and that there exists an optimal concentration for maximal muscular performance as illustrated in Figure 1.1. Reid proposed that the contractile changes mediated by RONS may involve several molecular targets, and that optimal contractility may be dictated by an oxidative state that optimises the function of all proteins involved in contraction (Reid 2001).
Fig. 1.1 Biphasic effect of RONS on muscle force production. Adapted from (Reid 2001). Model demonstrating the biphasic effect of RONS on skeletal muscle force production. In state (2) (basal) muscle exists in a relatively reduced state, which is not optimum for contraction. A decrease in redox state (1) results in a movement away from the optimum, resulting in lower force production. A moderate increase in redox state (3) is optimal for muscle contraction. Any further increase in oxidation (4) moves the cellular redox state further away from optimal, resulting in a decrease in force production.

### 1.3.3 RONS and the cellular damage hypothesis of muscle fatigue

Originally, RONS were thought to be purely destructive molecules and it was proposed that increased RONS production during skeletal muscle contraction caused oxidative damage leading to loss of contractile performance. Indeed, there is evidence that sustained intense exercise that results in high RONS concentrations can result in irreversible damage to both proteins and lipid membranes in both *in vitro* and *in vivo* models (Dillard *et al.* 1978;
Davies et al. 1982; Jackson et al. 1985; Alessio et al.; Duthie et al. 1990; Haycock et al. 1996; Giuliani and Cestaro 1997). This view linking RONS and muscle damage has been reinforced by the observation that RONS accumulation is associated with a wide range of diseases, many of which are associated with muscle fatigue, including coronary obstructive pulmonary disease (Rahman et al. 1996; Tager et al. 2000), coronary heart disease (Wolfram et al. 2003; Chen and Mehta 2004), Parkinson’s disease (Cassarino and others 1997; Bloomer et al. 2008; Henchcliffe and Beal 2008) and muscular dystrophy (Murphy and Kehrer 1989; Disatnik et al. 1998). In the case of muscular dystrophy, for instance, muscle dysfunction has been shown to be related to higher levels of oxidative stress measured by a number of biomarkers including protein carbonyls, malondialdehyde (MDA), as well as glutathione disulphide (Murphy and Kehrer 1989; Rando et al. 1998; Rando 2002).

However, the cellular damage hypothesis of RONS-mediated muscle fatigue has been challenged on the basis that muscle cells can generate RONS rapidly during exercise associated with little or no oxidative damage (Jackson et al. 1985; O’Neill et al. 1996). Also, muscular fatigue is generally a rapidly reversible phenomenon, whereas cellular damage is not. Furthermore, muscular fatigue has been demonstrated to occur in response to low-to-moderate RONS production and in the absence of irreversible cellular damage (Jackson et al. 1985; O’Neill et al. 1996; Margaritis et al. 1997; Akova et al. 2001; Wilber et al. 2004; Morillas-Ruiz et al. 2005). Although the cellular damage hypothesis may explain some cases of muscle fatigue, it does not explain how RONS mediate muscle fatigue in situations where little or no irreversible damage occurs. For this reason,
mechanisms by which RONS may mediate muscle fatigue independently of cellular damage have been proposed as discussed in the next section.

1.4 REVERSIBLE PROTEIN THIOL OXIDATION EFFECTS ON MUSCLE FUNCTION AND MUSCLE FATIGUE

There is now evidence to suggest that RONS play multiple regulatory roles independent of oxidative damage (Reid 2001; Jones 2006; Ferreira et al. 2008; Jones 2008; Powers and Jackson 2008; Reid 2008; Powers et al. 2011) thus raising the question of the nature of the mechanisms involved. One mechanism by which RONS could control muscle function is through the reversible oxidation of protein thiols as this can cause a change in protein structure and therefore its function. Protein thiol oxidative modification is a physiologically reversible process since thiols can be converted back to their reduced form through the action of enzymes (including glutaredoxin or the peroxiredoxins (Eaton 2006).

There is considerable *in vitro* evidence to suggest that numerous proteins are sensitive to thiol oxidation, including ion transport proteins (Liu and Pessah 1994), receptors, signal transduction kinases, phosphatases, transcription factors and contractile proteins (Paget et al. 2003; Ferreira and Reid 2008; Reid 2008; Winterbourn and Hampton 2008). It follows that RONS have the potential to affect the activity of proteins directly involved in muscle function as well as act indirectly through their effects on intracellular signaling pathways (Reid 2001; Ji 2007), either through thiol oxidation of kinases and phosphatases (Groen et al. 2005), or through oxidative thiol modifications to upstream effector proteins (Nishida et al. 2000).
Indirect evidence implicating protein thiol oxidation in muscle fatigue comes from in vitro studies of isolated muscle fibres showing that thiol oxidants, such as hydrogen peroxide, can increase fatigue, while thiol reducing agents such as dithiothrietol can attenuate fatigue (Andrade et al. 1998). This notion that protein thiol oxidation may influence muscle function and contribute to muscle fatigue is further supported by the observation that muscle performance is most consistently improved by anti-oxidants that oppose thiol oxidation (Ferreira and Reid 2008). For example, N-acetylcysteine (NAC), a thiol anti-oxidant and thiol donor that supports GSH re-synthesis (Ruffmann and Wendel 1991), attenuates muscle fatigue both in vitro and in vivo (Shindoh et al. 1990; Khawli and Reid 1994; Supinski et al. 1997; McKenna et al. 2006).

The primary mechanism by which NAC is thought to improve sustained muscular contraction is through its ability to act as a thiol donor to increase GSH re-synthesis and maintain the reduced thiol status of the cell (Meister and Anderson 1983; Ruffmann and Wendel 1991; Ji et al. 1993; Medved et al. 2004; Ferreira et al. 2008). NAC has been shown to reduce fatigue in rodents (Shindoh et al. 1990) as well as in humans in a variety of muscles. For example, Reid and colleagues reported a 15% increase in muscular force during fatiguing electrical stimulation of the tibialis anterior (Reid et al. 1994), Travaline and co-workers showed a 62% increase in time to exhaustion during diaphragm contractions (Travaline et al. 1997), and Medved and colleagues demonstrated a 26% increase in time to exhaustion during sustained high intensity bicycle ergometer exercise (Medved et al. 2004). However, NAC does not appear to attenuate muscle fatigue at exercise intensities near maximum muscular contraction. For example, Matuszczak and
colleagues demonstrated that NAC caused no increase in performance during sustained maximal efforts during hand grip exercise (Matuszczak et al. 2005). Similarly, Medved and colleagues reported no ergogenic effect when subjects performed repeated exercise bouts at 130% VO_{2}max and 45 second intervals (Medved et al. 2003). However, a study conducted by Lands and co-workers showed that three months of loading with a dietary whey based cysteine donor resulted in a 35% increase in lymphocyte GSH and a 13% increase in peak power and 13% increase in 30-second total work capacity relative to placebo (Lands et al. 1999).

1.4.1 Examples of protein thiol redox targets implicated in muscle fatigue and optimum contractility

Although there is considerable indirect evidence that protein thiol oxidation mediates RONS-induced muscle fatigue and optimal contractility, the target proteins are still largely unknown. Nevertheless, based on work performed with isolated muscle proteins, many proteins involved in contraction have been shown to be sensitive to protein thiol oxidation and therefore have the potential to be the targets mediating the effect of RONS on muscle contractile performance (Cassarino and others 1997; Bonne et al. 1998; Reid 2001; Colavitti and Finkel 2005; Powers and Jackson 2008).

There has been speculation that the ryanodine-sensitive Ca^{2+} release channel is involved in causing muscle fatigue (Abramson and Salama 1989; Favero 1999; Reid 2001; Moopanar and Allen 2006). Although the opening and closing of the sarcoplasmic reticulum ryanodine release channel is thiol redox sensitive (Abramson and Salama 1989), Moopanar and Allen have reported conditions where RONS do not affect sarcoplasmic reticulum
calcium release or the maximum Ca\(^{2+}\) activated force, but increase myofibrillar Ca\(^{2+}\) sensitivity (Moopanar and Allen 2005; Moopanar and Allen 2006). This would imply a role for the structural and contractile proteins. Support for the involvement of these proteins comes from some biochemical and structural studies performed in vitro which have provided evidence that RONS can alter both the structure and spatial organisation of structural proteins (Dalle-Donne et al. 2003; Dalle-Donne et al. 2003) as well as altering their contractile function (Mishima et al. 2005; Moopanar and Allen 2006) without affecting internal calcium levels. Furthermore, free radical scavengers like NAC and GSH have been shown to reduce fatigue, not by changing calcium levels, but through a direct effect on acto/myosin interaction (Diaz et al. 1994; Cooke and Cooke 2007; Murphy et al. 2008). For these reasons, structural and contractile proteins such as myosin, actin, troponin and tropomyosin are likely targets of RONS-mediated muscle fatigue (Moopanar and Allen 2005; Moopanar and Allen 2006; Smith and Reid 2006).

Myosin is a major contractile protein in skeletal muscle (Yates et al. 1983). It converts chemical energy into mechanical work and drives muscle contraction. Myosin is susceptible to a variety of oxidative modifications including thiol oxidation (Graceffa and Seidel 1980; Bhoite-Solomon et al. 1992; Hanan and Shaklai 1995; Lund et al. 2008) and it is well established that a variety of oxidative modifications of myosin occur in a number of diseases such as chronic heart failure (Seddon et al. 2007; Lund et al. 2008), diabetes (Aragno et al. 2004; Dursun et al. 2005; Oh-Ishi et al. 2007), aging (Thompson et al. 2006), and ischemic reperfusion injury (White et al. 2005).
Myosin contains over 40 thiol groups of which 12 or 13 reside on each of the two myosin globular heads (Reisler et al. 1982). Each globular head of myosin contains the binding sites for ATP and actin, is responsible for myosin ATPase activity during muscle contraction, and contains the highly reactive thiol groups SH1 (cys 707) and SH2 (cys 697). These thiol groups are at, or very close to, the fulcrum point where the neck of the myosin head swings back to generate force during ATP hydrolysis (Uyeda et al. 1996). Their oxidation affects myosin ATPase activity (Yamashita et al. 1964; Graceffa and Seidel 1980; Tiago et al. 2004) and causes a marked loss of contractile function (Sekine and Yamaguchi 1963; Burke et al. 1976; Crowder and Cooke 1984; Hiratsuka et al. 1998; Lowe et al. 2001; Ooizumi and Xiong 2004). These thiol groups are candidates for oxidative modification and potentially involved in skeletal muscle contractile function, and their roles in RONS-mediated skeletal muscle fatigue in vivo warrant further investigation.

While it is myosin that drives muscular contraction, it is actin that serves as the structural framework through which myosin generates work. There is evidence that actin is also sensitive to thiol redox modification (Hinshaw et al. 1991; Omann et al. 1994; Mocali et al. 1995) and the exposed thiol group (cys-374) has been implicated (Chai et al. 1994). These findings suggest that thiol redox state of actin is also a likely target for RONS-mediated muscular fatigue and associated effects on contractile performance.

Oxidative modification of thiol groups can also affect tropomyosin and troponin. Williams and Swenson (1982) showed that disulphide bonds at cys-190 on the tropomyosin molecule has an inhibitory effect on the myosin ATPase activity (Williams and Swenson 1982). Troponin has also been shown to have highly reactive cys-84 and cys-35 residues which are
susceptible to redox modification and capable of affecting skeletal muscle activity (Putkey et al. 1993). Recently S-glutathionylation of troponin I has been shown to increases Ca$^{2+}$ sensitivity in fast-twitch isolated muscle fibres of rats and humans and cys 133 has also been implicated as thiol redox sensitive (Mollica et al. 2012). In addition, reversible oxidation of cysteine 98 on troponin reduces the binding affinity of troponin for actin, thereby destabilising the troponin-actin relationship resulting in a loss of force production (Pinto et al. 2011). However, it is still unknown whether troponin and tropomyosin are thiol redox modified in response to skeletal muscle fatigue in humans in vivo.

The glycolytic enzymes as well as glycogen phosphorylase, all of which play an important role in energy production during high intensity physical exercise, are also potential targets of RONS since their activities are affected by changes in their level of thiol oxidation. In this respect, glycogen phosphorylase has been shown to undergo reversible thiol oxidative modification in vitro, and this modification can result in the inactivation of this enzyme (Gratecos et al. 1977; Ziegler 1985; Cappel and Gilbert 1986).

1.5 MEASUREMENT OF THE LEVEL OF PROTEIN THIOL OXIDATION AS A BIOMARKER OF OXIDATIVE STRESS AND AS A MEANS TO IDENTIFY THE TARGETS OF OXIDATIVE STRESS

It is clear from the above discussion that the structure and function of a large number of proteins are sensitive to protein thiol oxidation. What is still unclear, however, is the extent to which the level of thiol oxidation of these proteins responds to physiological and pathophysiological oxidative challenges in vivo. Due to the extremely short half life of
RONS and their highly reactive nature, their direct measurement is extremely difficult. A common approach is the indirect measurement of oxidative stress by the quantification of the molecular products formed by the reaction of RONS with various cellular structures (Fisher-Wellman and Bloomer 2009).

In general, most of the research concerned with assessing oxidative stress in vivo have focused on measuring changes in the biomarkers of oxidative stress associated with cellular damage (Shacter 2000; Dalle-Donne et al. 2006; Fisher-Wellman and Bloomer 2009). These analytical techniques generally measure irreversible oxidative damage to cellular structures either by measuring oxidation products directly (eg carbonyl assay for oxidised proteins) or the resultant degradation products (eg malondialdehyde for lipid peroxidation) (Halliwell and Whiteman 2004). These techniques have been used extensively as biomarkers of oxidative stress in tissue and plasma of animals (Disatnik et al. 1998; Kaczor et al. 2007) and humans (Dillard et al. 1978; Margaritis et al. 1997; de Zwart et al. 1999; Miyazaki et al. 2001; Thompson et al. 2003; Kadiiska et al. 2005). One limitation with assessing oxidative stress by measuring the levels of the aforementioned biomarkers is that protein thiol oxidative modifications may occur at RONS concentrations lower than that associated with irreversible cellular damage (Jones 2008) and so the RONS concentrations necessary for protein thiol oxidation would not be detected by the use of such techniques.

The levels of glutathione (GSH) and the ratio of glutathione to glutathione disulphide (GSH/GSSG) have also been used as biomarkers of oxidative stress. However, these biomarkers may not provide an accurate measure of muscle oxidative state since GSH can be released to the extra-cellular space (Sen 1998) and imported from plasma by exercising
skeletal muscles via the γ-glutamyl cycle. Furthermore, GSH can be synthesised by the liver and released in response to elevated plasma glucagon and vasopressin levels during exercise (Ji et al. 1993).

Since the physiological and pathophysiological effects of oxidative stress depend to a large extent on the effect that RONS have on protein thiols, the measurement of the level of thiol oxidation of proteins may provide a more relevant measure of oxidative stress in many research applications. In addition, it will provide information specific to the effect of RONS on protein thiols and help to identifying the protein targets of oxidative stress. Unfortunately, the current understanding of the biological significance of reversible protein thiol oxidation in tissue has been hampered by a lack of suitable analytical techniques (Makmura et al. 2001; Reid 2008; Hawkins et al. 2009; Powers et al. 2010), which has been limited by technical challenges which, in part, are a consequence of the highly reactive nature of protein thiols (Jacob et al. 2003). The many technical challenges associated with the measurement of protein thiols and determining their oxidation state in tissue are discussed in the next section.

1.6 THE MEASUREMENT OF REDUCED AND OXIDISED PROTEIN THIOLS: GENERAL PRINCIPLES AND TECHNICAL CHALLENGES

Since oxidation of protein thiols may occur at RONS concentrations lower than that required to cause irreversible oxidative damage (Jones 2006; Jones 2008), the measurement of the oxidation state protein thiols of a complex tissue sample may provide a more sensitive biomarker of oxidative stress than other biomarkers that measure oxidative
damage such as the carbonyl or MDA assay. Furthermore, the measurement of the level of protein thiol oxidation of total proteins in a complex tissue sample provides a snapshot that captures the extent of thiol oxidation in different physiological or pathological states and is one way to gain insight into the biological significance of protein thiol oxidation in vivo. This section will focus on some of the technical challenges encountered with generating physiologically relevant data in tissue.

1.6.1 Sample collection and storage

One of the difficulties encountered in establishing a clear link between protein thiol oxidation and muscle function in health and disease, is the many technical problems associated with the reliable and quantitative measurement of protein thiol oxidation in tissue. For example, protein thiols are highly reactive and oxidation of thiol groups by ambient molecular oxygen can occur rapidly and spontaneously (Creighton 1984; Hansen and Winther 2009) due to the abundance of oxygen in the atmosphere. As a consequence there is a possibility that artefactual oxidation will occur during tissue sampling, storage and assaying procedures.

The first challenge is therefore to prevent artefactual oxidation during sample collection. An extensive review of the literature revealed no study that has described how rapidly protein thiol oxidation occurs after tissue sampling. However, because of the hypoxic stress experienced by a tissue following its removal from the body and the highly reactive nature of protein thiols, there is potential for RONS production and protein thiol oxidative modification if samples are not rapidly snap frozen in liquid nitrogen immediately after sampling (Hansen and Winther 2009). Furthermore, enzyme activity may not be fully in-
activated at -20°C, meaning there is a possibility of unwanted side reactions if samples are not stored at -80°C (Passonneau and Lowry 1993).

1.6.2 Sample preparation

Sample preparation also poses its own challenges for the precise determination of the level of thiol oxidation of proteins. Since the analysis of reduced and oxidised protein thiols involves the use of labelling reagents and in some cases the use of blocking reagents targeting thiol groups, it is important that the protein thiol groups be accessible to the reagent. Mechanical disruption of tissue and denaturation of proteins has been used to ensure protein thiol groups are accessible to labelling reagents. The main challenge here is avoiding artefactual oxidation and thiol disulfide exchange reactions during tissue preparation (Hansen and Winther 2009; Hill et al. 2009).

Artefactual oxidation and thiol disulfide exchange reactions can be minimised by preparing tissue sample under acidic conditions and low temperatures (Hansen and Winther 2009; Hill et al. 2009). A low temperature is advantageous as it slows the rate of oxidative reactions. This is normally achieved by including acetone in the homogenisation buffer as an acetone solution can remain liquid even at -20°C (Passonneau and Lowry 1993). Acid protein precipitants such as trichloroacetic acid (TCA) are advantageous because protein thiols can be separated from low molecular weight thiols such as GSH and free cysteine which have the potential to interfere with protein labelling or participate in thiol-disulfide exchange reactions. The effectiveness of acidic precipitants such as TCA, perchloric acid (PCA), and sulfosalycilic acid (SSA) in preventing thiol oxidation has been tested, and TCA has been shown to be more effective than SSA and PCA at both 0°C and room
temperature, which is not surprising given that PCA is a strong oxidant (Rossi et al. 2002). A further important advantage of acidic precipitants such as TCA is that enzymes and cellular reductants such as NADPH or NADH which could cause unwanted side reactions are inactivated (Passonneau and Lowry 1993).

### 1.6.3 Quantitative protein solubilisation and saturation labelling

The solubilisation of the protein pellet after acid treatment presents another important challenge. Solubilisation of tissue proteins, particularly that of muscle, is notoriously difficult (Babu et al. 2004; Thierry Rabilloud et al. 2006; Bodzon-Kulakowska et al. 2007). Incomplete solubilisation of proteins can lead to a loss of accuracy if solubilised proteins are not representative of tissue and a loss of precision if the degree of solubilisation of individual proteins varies from sample to sample. The challenge here is to identify the conditions that optimise solubilisation (use of detergents, long incubation time, high pH, high temperature, and other tissue dispersion techniques such as sonication, vortexing, and homogenisation) without promoting protein thiol oxidation or interference with other reagents.

Neutral or near neutral pH environments are typically used to solubilise protein after acid precipitation. However, at these pH levels, artefactual oxidation by molecular oxygen and thiol-disulfide exchange can occur, thus confounding the assay results. This is an important issue as illustrated by the pH dependency of the oxidation of GSH. Rossi and co-workers demonstrated almost complete oxidation of GSH within 10 minutes when TCA treated samples were restored back to pH 8.5, but the oxidation was minimised to less than 5% if the pH was restored to 7.25 (Rossi et al. 2002).
To minimise artefactual oxidation during solubilisation, saturation labelling can be performed during the solubilisation process to alkylate thiols and therefore protect them from oxidation. This procedure is based on the premise that labelling reactions with an alkylating reagent occur more rapidly than oxidative reactions, but this is specific to the thiol labelling reagent used. While saturation labelling may prevent artefactual oxidative side reactions, it has one potential disadvantage in that it may further inhibit the solubilisation process (Patton 2000; Riederer et al. 2008). For instance, Shaw and co-workers reported that the large hydrophobic groups which is part of the CyDye DIGE Fluors used in their assay, can lead to a loss of solubility of protein of approximately 25% (between 1% and 40%) during labelling. They also reported that the losses in proteins during saturation labelling were more prevalent in high molecular weight proteins, thus creating a bias toward the lower molecular weight proteins (Shaw et al. 2003).

Since detergents disrupt hydrophobic interactions and enhance protein solubilisation and extraction, their use in protein solubilisation with or without saturation labelling is generally advocated. Detergents, such Triton X-100, Tween, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) have been shown to improve protein solubilisation (Riederer et al. 2008). Riederer and co-workers have examined the effect of a number of detergents including Triton X-100, Tween and CHAPS on protein labelling, solubilisation, and recovery using infra-red maleimide dyes and desalting columns to remove un-reacted reagents. They reported that Triton X-100 and Tween are the most effective (approx 30-50% recovery), with CHAPS being only marginally better than no detergent at all. Furthermore, they noted differences in labeled
protein solubility between mouse and human brain, indicating that the type of tissue sample may influence labelling, solubility and/or recovery of protein (Riederer et al. 2008).

The use of detergents is also advantageous because their denaturing properties enable protein thiols to be more effectively labeled, particularly if saturation labelling is performed. SDS is highly efficient at solubilising hydrophobic membrane (Churchward et al. 2005) proteins and is therefore especially useful for biological tissue samples. However, some caution is required with the use of detergents such as SDS as they may interfere with some alkylating reagents and downstream processes such as labelling, reduction, 2-D electrophoresis and protein assay. In some circumstances this can be avoided by careful manipulation and dilution of the detergent and other reagent and the use of appropriate detergent-compatible assays (Riederer et al. 2008).

Under conditions where saturation labelling is used, it is important to remove un-reacted reagent. Unfortunately, techniques to remove un-reacted reagent can cause further loss of protein. Riederer reported protein loss of between 60-75% of starting material (20-45% protein recovery) using desalting columns after saturation labelling with maleimide dyes. Another approach that minimises protein loss is to remove un-reacted reagent by precipitating proteins with the addition of ethanol or methanol followed by the removal of un-reacted dye in the supernatant after centrifugation (Riederer et al. 2008).

Irrespective of the composition of the medium used to solubilise protein thiols, the additional use of physical techniques involving homogenisation using rotor-stator homogenisers, vortexing and sonication has been shown to be effective to help break down
solid particles and improve solubilisation. (Thierry Rabilloud et al. 2006; Bodzon-Kulakowska et al. 2007). Used in combination with detergents, physical techniques can enhance the solubilisation process. However, these techniques may increase heat and/or exposure to oxygen and have the potential to cause artefactual oxidation.

It follows from above that conditions that optimise solubilisation (use of detergents, long incubation time, high pH, high temperature, and other physical techniques such as sonication, vortex, homogenization), can cause artefactual protein thiol oxidation or interfere with other reagents. In contrast, incomplete solubilisation and recovery of proteins after labelling can lead to a loss of accuracy if solubilised proteins are not representative of tissue and a loss of precision if the degree of solubilisation of individual proteins varies from sample to sample. For these reasons, careful attention must be paid to optimise these procedures for the assay to be valid (Riederer et al. 2008).

1.6.4 Measurement of free (reduced) protein thiols

The measurement of free (reduced) thiol content has been used to indirectly estimate changes in reversible protein thiol oxidation in a variety of research settings (Mirabelli et al. 1988; McArdle et al. 1999; Balcerczyk et al. 2003; Prakash et al. 2004; Varsanyi et al. 2004; Dursun et al. 2005; Eaton 2006). Free protein thiols can be measured using a variety of techniques that in general use reagents to react with the free thiols of proteins, with the underlying assumption that the particular reagent is specific for protein thiols. A commonly used reagent for thiol measurement is Ellman’s reagent (5, 5’-dithiobis-2-nitrobenzoic acid, DTNB) (Ellman 1959). Methods based on this reagent uses an excess of DTNB over thiols, resulting in the thiol disulfide exchange reaction generating TNB (5-
thio-2-nitrobenzoic acid) which can be quantified spectrophotometrically at 412 nm (Eyer et al. 2003). The reaction is quick and does not require standards for quantitative measurement. This may explain why Ellman’s reagent has been widely used for the quantification of reduced protein thiols (Hansen et al. 2007).

There are several potential limitations with using Ellman’s reagent to examine protein thiol oxidative modifications. One limitation is the lack of sensitivity, with a detection limit of about 3 μM (Riener et al. 2002; Hansen et al. 2007), making subtle changes in the redox state in physiological changes difficult to detect. In addition, because the rate of the reaction is affected by pH and the pKa of the thiol group (Riddles et al. 1983), a pH of 7.0 is usually used when assaying thiols. Consequently, the Ellman’s reagent is added after solubilisation at pH 7.0, but this increases the potential for artefactual oxidation, which in turn can result in the underestimation of protein thiols.

Alternatives to Ellman’s reagent are fluorescent thiol labelling reagents as they are an order of magnitude more sensitive. These reagents can be added during solubilisation, therefore reducing the possibility of oxidative reactions during solubilisation. Furthermore, since after reacting with the thiol groups of proteins, these labelling reagents remain bound to proteins, further separation techniques such as 1D or 2D SDS PAGE can be used to identify individual proteins (Riederer and Riederer 2007; Riederer et al. 2008). Many fluorescent thiol reactive probes are available, including thiol reactive reagents such as maleimides, iodoacetamides, iodoacetate and thiosulfates which have varying specificities for thiol groups. A commonly used labelling reagent is monobromobimane (Rossi et al. 2002; Petrotchenko et al. 2006), which is a fluorescent reactive aldehyde which reacts with thiols.
to form the highly reactive thioether (Kosower and Kosower 1995; Hansen and Winther 2009). Monobromobimane has several limitations including susceptibility to photodegradation, cross reacting with thiol based reductants and phosphines, and a lack specificity for thiol groups (Graham et al. 2003; Hansen and Winther 2009). In contrast, maleimides react quickly and irreversibly with thiol groups, and are relatively stable (Baldwin and Kiick 2011).

Irrespective of the sensitivity of the thiol reagent used, there is a major limitation with measuring the free protein thiols of tissues as a means to estimate changes in protein thiol oxidation. Most intracellular proteins exist in predominantly reduced state (Paget et al. 2003), so subtle changes in the oxidation state of protein thiols are difficult to detect against a substantial background signal. For example, if a tissue sample, has approximately 90% of protein thiols reduced and 10% oxidised, a 50% increase in oxidised thiols (from 10% to 15%) would represent only a 5% change in free protein thiols (90% to 85%). In this context, biological variability and assay imprecision may make it difficult to detect such small fractional changes when only free thiol status of a protein sample is measured.

Another important limitation with the measurement of free thiols is that it assumes that increases in the formation of oxidised thiols will directly result in a decrease in free thiols. However, changes in free thiol content of a protein sample can result from a number of oxidative modifications, not just reversible protein thiol oxidation. Indeed, protein thiols can be oxidised to biologically irreversible products such as protein carbonyls or the sulfonic acid derivative (Barford 2004; Eaton 2006; Turell et al. 2009). Unless additional control experiments are undertaken, it is therefore uncertain that a measured decrease in
free protein thiols would reflect an increase in reversible protein thiol oxidation (Eaton 2006).

A further complication with using changes in free thiol as an indirect measure of the level of protein thiol oxidation in tissue is that protein thiol content can vary between tissues and physiological conditions, which presumably reflect differences in the protein composition. Therefore, in situations where protein composition is affected, for example in response to chronic disease, differences in reduced thiol content may reflect changes in the proportion of thiol containing proteins rather than reflecting changes in reversible the level of protein thiol oxidation.

It is clear from the above arguments that in many research settings, the direct measurement of protein thiols in their oxidised state provides researchers valuable information regarding oxidative modifications that cannot be gained from the measurement of free thiols alone.

1.6.5 Measurement of oxidised protein thiols

Given the many limitations with measuring reduced thiols as an indirect means to evaluate the level of protein thiol oxidation of a tissue sample, a number of methods have been developed to measure oxidised thiols. One method is to calculate oxidised thiols indirectly by measuring free and total thiols separately, and then calculating oxidised thiols by subtracting free thiols from total thiols after standardising both measurements according to a protein assay (Medved et al. 2004). Another method is to measure oxidised thiols directly by using alkylating or labelling reagents to irreversibly quench free thiols, followed by the use of a reducing agent to convert oxidised thiols to free thiols, and then the use of a
thiol labelling reagent to react with the newly formed free thiol groups. The measurement of oxidised thiols has the further advantage of improving the detection limit by reducing the background signal when a tissue exists in a predominantly reduced state as they do in biological tissues under normal physiological conditions.

A number of reagents such as N-ethylmaleimide (NEM), iodoacetic acid, and iodoacetamide have been used to alkylate protein thiols. NEM is commonly used as it is highly reactive and relatively specific for thiols when used at concentrations of 1-20 mM and at pH 7.0 or less (Brewer and Riehm 1967). It reacts significantly more rapidly than iodoacetic acid or iodoacetamide (Brewer and Riehm 1967 1150; Rogers et al. 2006; Hansen and Winther 2009), with typical incubation times of one to five minutes (Lind et al.). Furthermore, iodoacetic acid or iodoacetamide require a pH above 8.0 for effective alkylation to occur (Rodgers et al. 2006), thus increasing the potential for artefactual oxidation reactions as discussed earlier.

1.6.6 Reduction of oxidised protein thiols

Following the blocking or labelling of free protein thiols with an alkylating reagent, the next step in the measurement of oxidised protein thiols is to use an appropriate reducing agent. Although a variety of reducing agents are available, there are complications associated with their use.

Thiol containing reducing reagents such as dithiothreitol (DTT) and β-mercaptoethanol, have the advantage of being specific to oxidised protein thiols (Hansen et al. 2009). DTT has the advantage that it is a stronger reductant than β -mercaptoethanol and can be used at
lower concentrations to avoid mixed disulphides (Cleland 1964). β-Mercaptoethanol also has a pungent smell which restricts its use to within a fume hood. A disadvantage, however, with both of these thiol-based reducing reagents is that they are particularly sensitive to oxidation and are generally used at a pH above seven, which also increases the potential for artefactual oxidation (Hansen and Winther 2009). A further disadvantage is that the thiols of the reductant compete directly with protein thiols for attachment to thiol labelling reagents. Removal of reducing agents prior to labelling is possible but may cause re-oxidation of protein thiols. This can be overcome by swamping the reductant with high concentrations of the thiol labelling reagent, but thiol labelling reagents can be expensive, so this method may not be practical. Furthermore, high concentrations of thiol labelling reagents can increase the potential for non-specific binding to non-thiol structures, thus causing an over-estimation of oxidised thiols.

Phosphines can be used to reduce oxidised protein thiols as alternatives to thiol reducing reagents. They are generally more stable than thiol based reductants and generally less reactive toward certain thiol labelling agents. This has the advantage that the reducing agent does not need to be removed for labelling to take place (Hansen and Winther 2009). Tris(2-carboxyethyl)phosphine (TCEP), is an effective water soluble reducing agent that is widely used, but there are some caveats with its use. TCEP is generally more stable than thiol based reductants, but it has been reported to be unstable in phosphate buffers (Burns et al. 1991). TCEP is also highly acidic, so care must be taken to ensure adequate buffering. TCEP can also interact with maleimide labelling reagents and reduce labelling efficiency (Burmeister et al. 1999), so care must be taken to ensure complete labelling of protein thiol groups in the presence of TCEP.
Another reducing agent that has been used is sodium borohydride (Hansen et al. 2007). Sodium borohydride can be removed relatively easily with the addition of acetone or acid. However, hydrogen is produced during the reaction, so foaming can result (Krull and Friedman 1967; Hansen et al. 2009), making the sample difficult to work with and possibly increasing the risk of side reactions. Another limitation with sodium borohydride is that it catalyses the hydrolysis of Asn and Gln amides as well as the peptide bonds which limits its use for mass spectrometry (Hansen and Winther 2009).

1.6.7 Protein thiol labelling

There are a number of thiol labelling techniques available, including methods based on radio-labelling, biotin-conjugation, and fluorescence. For complete labelling to take place, it is important to ensure appropriate pH and incubation times for alkylation to occur. A high reaction pH and long incubation period will increase the alkylation reaction, but will also increase the possibility of unwanted side reactions. Low pH and short incubation times will reduce the possibility of unwanted side reactions, but may result in incomplete labelling of thiols, so reaction rates and oxidation rates must be monitored carefully.

Biotin-based tagging techniques have been used to measure the intra-cellular thiol status of proteins (Hill et al. 2009). Thiols can be labeled directly with biotin-tagged reagents such as biotinylated IAM (BIAM) or biotinylated NEM (Bt-NEM), and the biotin signal can be assessed using western blotting, with the loss of the biotin signal proportional to the degree of thiol modification. However, some proteins, such as carboxylases have biotin covalently attached, giving false positive results that may be misleading. In contrast, fluorescent thiol
alkylating agents have the advantage that western blotting is not required, and the level of
alkylation can be detected based on the emission/excitation characteristics of the particular
fluorescent probe. Another advantage of fluorescent techniques is that they are generally
highly sensitive and have a large dynamic range.

1.6.8 *Ratio-metric dual labelling techniques*

The measurement of both free and oxidised protein thiols on the same tissue sample,
provides a ratio-metric measure which can further improve detection limits and sensitivity.
A ratio-metric measure offers greater sensitivity than simple quantitative measures because
the directional changes in reduced and oxidised protein thiols are in opposite directions and
therefore the magnitude of the change in the ratio is greater than the change in either
reduced or oxidised protein thiols alone (Bullen and Saggau 1999). This sensitivity is
increased at the extremes of reduction or oxidation. Since tissues exist in a predominantly
reduced state under physiological conditions (Paget et al. 2003), a ratio-metric measure has
far greater sensitivity than simple quantitative measures. Furthermore, if free and oxidised
thiols are measured independently, error can be introduced during assay procedures if
samples are not treated identically, and the two samples must be standardised for protein
concentration or tissue wet weight, which introduces another possible source of error.

1.6.9 *Use of SDS PAGE to evaluate the changes in the level of thiol oxidation of
individual proteins*

One dimensional SDS PAGE isolates a complex tissue sample into proteins of similar
molecular weight. This is particularly useful to allow for screening of possible protein
targets for reversible thiol modification, and allows for the quantification of the oxidation
state of a large number of individual protein bands simultaneously. The extent of thiol oxidative modifications resulting from an oxidative stress in tissue may be specific to individual proteins (Leichert et al. 2008). For this reason, isolating proteins using 1 D SDS PAGE increases the sensitivity to detect oxidative changes within a specific proteins beyond that achieved by global measures, since it eliminates the background signal from other proteins. Where oxidative changes have been determined, the protein band can be removed from the gel and the proteins in the band can be identified using mass spectrometry.

It should be noted that several proteomic methods have been developed to identify oxidative changes to specific target proteins (Eaton 2006; Leichert et al. 2008; Riederer et al. 2008). Since the thiol reactivity of proteins is specific to the protein in question, the oxidation state of a specific protein may not reflect the global state of total protein thiol oxidation and RONS concentration in tissue. As a consequence these techniques may not be suitable for use as a biomarker of oxidative stress, nor measure the response of tissue to pathological, physiological, or clinical intervention in vivo. Furthermore, these methods are technically difficult, time consuming and do not lend themselves to be used on a large number of samples.

1.7 SUMMARY

RONS are produced by skeletal muscle in response to exercise and their production has been implicated to be involved in many aspects of muscle function. Originally RONS were thought to be purely destructive molecules causing irreversible cellular damage. However,
there is now evidence to suggest that RONS play multiple regulatory roles independent of cellular damage. One mechanism by which RONS may operate to control muscle function is through the reversible oxidation of protein thiols which has the potential to affect many aspects of muscle function. However, the response to protein thiol oxidation to exercise has not previously been demonstrated \textit{in vivo}.

Research into oxidative stress, and in particular, the effect exercise on the level of protein thiol oxidation, has, to a large extent, used markers of oxidative stress that involve measurements related to cellular damage. However, measurements of cellular damage may not reflect the effects that RONS have on protein thiols. As a consequence, research into the effect of protein thiol oxidation into muscle function has been hampered by a lack of suitable analytical techniques. This is due, in part, to the highly reactive nature of protein thiols and the fact that their accurate measurement should incorporate their quantification in both their reduced and oxidised forms, and must overcome many confounding issues.

\section*{1.8 STATEMENT OF THE PROBLEM AND HYPOTHESES}

Given the potential benefits of a dual labelling assay for the measurement of reduced and oxidised protein thiol groups, one of the primary objectives of this thesis was to develop such a technique, taking into account all of the aforementioned limitations associated with tissue sampling, protein extraction, protein labelling and assay sensitivity and specificity. The next objective was to apply this technique to investigate the changes in the level of thiol oxidation of muscle proteins associated with exercise in animal and human models since protein thiol oxidation has been implicated in a wide range of cellular functions.
related to exercise. In particular these models were developed with the aim of investigating a possible link between protein thiol oxidation and muscle fatigue. Finally, using 1 D SDS PAGE and mass spectrometry the aim was to investigate the extent of thiol oxidation of specific proteins that have been identified \textit{in vitro} to be likely targets, including myosin, actin, troponin and glycogen phosphorylase.

The aims and hypothesis of each chapter are as follows:

**Chapter 2**

The aim of this chapter is to develop a ratio-metric technique that measures both free and oxidised protein thiols within the same complex biological tissue sample and to further extend the technique for the measurement of the level of thiol oxidation of isolated protein bands. This is referred to as the “dual labelling” technique.

**Chapter 3**

The response of the level of protein thiol oxidation to exercise has yet to be measured under physiological conditions. So the aim of chapter three is to apply the “dual labelling” technique to an animal model of exercise and to test the following hypotheses:

1) Moderate intensity exercise which does not result in fatigue is not associated with an increase in the level of reversible protein thiol oxidation.

2) High intensity exercise resulting in significant muscle fatigue causes an increase in the level of protein thiol oxidation.

3) The level of protein thiol oxidation returns progressively to resting levels within 60 minutes of high intensity exercise.
Chapter 4

The aim of chapter four is apply the “dual labelling” technique to a human model of exercise and to test the hypotheses that:

1) 30 seconds maximal sprint cycling causes an increase in the level of thiol oxidation of proteins in the *vastus lateralis* muscle.

2) The level of protein thiol oxidation returns to resting levels within 40 minutes of passive recovery.
CHAPTER 2

A FLUORESCENT DUAL LABELLING TECHNIQUE FOR THE QUANTITATIVE MEASUREMENT OF REDUCED AND OXIDISED PROTEIN THIOLS IN TISSUE SAMPLES
ABSTRACT

Oxidative stress can result in the reversible oxidation of protein thiols. Since the activity of numerous proteins is sensitive to thiol oxidation, this has the potential to affect many cellular functions. The following describes a highly sensitive, quantitative labelling technique that measures global and specific protein thiol oxidative state in skeletal muscle tissue. The technique involves labelling the reduced and oxidised protein thiols with different fluorescent dyes. The resulting sample is assayed using a 96-well plate fluorimeter, and individual protein bands can be separated using SDS PAGE. The results show that artefactual oxidation during sample preparation and analysis has the potential to confound results and techniques to prevent this are described. The technique was tested by analysing the muscles of mdx and c57 mice, and the results show that the muscles of mdx mice were significantly (p < 0.05) more oxidised (13.1 ± 1.5 % oxidised thiols) than those of c57 mice (8.9 ± 0.7 % oxidised thiols). This technique provides an effective means to measure the extent to which oxidative stress affects the oxidation of protein thiols in biological tissues.
2.1 INTRODUCTION

Reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals, and reactive nitrogen species (RNS), such as peroxinitrite, have a diverse range of actions in cells (Eaton 2006). For instance, these reactive species can disrupt cellular function by irreversibly damaging proteins. ROS and RNS (RONS) can also affect protein function by oxidising critical cysteine residues (thiols). Although oxidation has the potential to be irreversible (to sulfonic acid), it is the biologically reversible oxidations which are of interest. Biologically relevant oxidations include the formation of sulfenic acid and nitrosylation as well as the formation of disulfide bonds with adjacent thiols on the same protein, or with glutathione (glutathionylation), or with the thiols of other proteins (Klatt and Lamas 2000; Di Simplicio et al. 2003). These are physiologically reversible processes, since the oxidised form can be converted back to the reduced thiol form through the action of enzymes such as glutaredoxin (Eaton 2006; Gallogly et al. 2007).

The activity of numerous proteins has been demonstrated to be sensitive to thiol oxidation, including ion transport proteins, receptors, signal transduction kinases, phosphatases, transcription factors and contractile proteins (Ferreira and Reid 2008; Reid 2008; Winterbourn and Hampton 2008). Consequently, protein thiol oxidation has the potential to influence many cellular functions, and has been associated with many diseases including schizophrenia (Do et al. 2009), Alzheimer’s (Yap et al. 2009), Parkinson’s (Qureshi et al. 2007), and diabetes mellitus (Dursun et al. 2005). Protein thiol oxidation has also been implicated as a causative factor in exercise-induced muscular fatigue (Reid 2008) and aging.
(Droge 2002). As a result, there is considerable interest in analytical techniques which can detect changes in the level of thiol oxidation of proteins in skeletal muscle.

Analytical methods such as the Ellman’s assay (Ellman 1959) and numerous other thiol reactive reagents (Eaton 2006) can measure the quantity of reduced thiols in a protein sample, with changes in protein thiol oxidation estimated from changes in reduced protein thiol content. However, changes in free thiol content of a protein sample can result from a number of oxidative modifications, not just reversible protein thiol oxidation (Eaton 2006). Unless additional control experiments are undertaken, it is therefore uncertain that a measured decrease in free protein thiols would reflect an increase in reversible protein thiol oxidation. Furthermore, proteins in tissue exist in a predominantly reduced state (Paget et al. 2003), so subtle changes in the oxidation state of protein thiols are difficult to detect against a substantial background signal.

Several factors limit the reliable and quantitative measurement of the level of protein thiol oxidation in tissue. Sample preparation can limit accuracy as tissue proteins can be difficult to solubilise (Unlu et al. 1997; Shaw et al. 2003; Babu et al. 2004; Riederer et al. 2008; Thierry 2009). In addition, protein solubilisation procedures may cause artefactual oxidation of protein thiols due to their highly reactive nature (Rossi et al. 2002; Rodgers et al. 2006; Hawkins et al. 2009).

Here we describe a fluorescent dual labelling technique for the direct quantitative measurement of both the reduced and oxidised protein thiols in skeletal muscle. A number of strategies are described to improve protein solubilisation and prevent artefactual
oxidation during extraction and analysis. The technique utilises a 96-well plate assay to
determine the global level of protein thiol oxidation of a complex protein sample.
Furthermore, it can be extended using techniques such as SDS PAGE to measure the level
of thiol oxidation of specific protein bands within a tissue sample.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Dystrophic male (mdx) mice and non-dystrophic control (c57) mice were purchased from
the Animal Resources Centre, Western Australia. Mice were housed in the pre-clinical
animal facility in accordance with the guidelines of UWA and NHMRC Animal Ethics.
For control tests, muscles samples were taken from the hind limb of male c57 mice. The
oxidation state may differ between control experiments due to the biological variation
between individual samples.

2.2.2 Materials

Ethanol (99.5%) and acetone (99.5%) were purchased from Redox chemicals. Double
deonised water was used throughout. Protein molecular weight standards were purchased
from BioRad, Australia. Unless otherwise stated, all chemicals and reagents were obtained
from Sigma Aldrich (Castle Hill, Australia).
2.3. DEVELOPMENT OF THE DUAL LABELLING TECHNIQUE FOR THE MEASUREMENT OF THE THIOL OXIDATION/REDUCTION STATE IN TISSUE PROTEINS

2.3.1 Sample Preparation

Weighed muscle samples were crushed with a pestle and mortar submerged in liquid nitrogen. Ice cold 20% TCA/acetone (20% w/v) was added, yielding a wet weight muscle concentration of 20 mg/ml. The sample was homogenised (Ultra-Turrax T25 - Rose Scientific) on maximum for 15 seconds to produce an even suspension, then incubated for at least one hour at -20°C. To remove TCA, a 50 μl aliquot was taken and 1.5 ml of acetone (pre-cooled to -20 °C) was added. The sample was vortexed, then centrifuged for five minutes at 8000 g at 4 °C. The supernatant was removed, leaving the protein pellet undisturbed. This washing procedure was repeated twice more, first with 1.5 ml of acetone, and then with 1.5 ml of ethanol.

2.3.2 Labelling of Reduced Thiols

After TCA removal, the resulting protein pellet was solubilised in 50 μl of SDS buffer (0.5% SDS, 0.5 M Tris, pH 7.0) containing 540 μM BODIPY FL-N-(2-aminoethyl) maleimide (FLm - Invitrogen). The protein pellet was solubilised by sonication (on ice) until the pellet was completely dispersed. Care was taken during sonication to avoid frothing. Samples were then centrifuged at 8000 g for five minutes (4°C) and the supernatant was retained. Then, another 50 μl of SDS buffer containing 540 μM FLm was added to the pellet, sonication and centrifuge were repeated as before and the entire pellet dispersed into a clear fluorescent solution. The supernatant was removed, and the two
supernatants were combined. The FLm labelling reaction was continued for 30 minutes at room temperature in the dark, as the fluorescent tags are light sensitive.

To remove excess FLm, 1.5 ml of ethanol pre-cooled to -20°C was added to the FLm-labeled protein extract, briefly vortexed, and incubated at -20°C for at least one hour to precipitate proteins. Samples were then centrifuged for five minutes (8000g, 4°C), and the supernatant was removed, leaving the resulting protein pellet undisturbed. The ethanol rinse was repeated, and the protein pellet was re-suspended in 100 μl of SDS buffer and vortexed until completely dissolved.

Samples were standardised according to their FLm (i.e. reduced protein thiol) concentration. With this approach, samples could be read at similar points along the fluorescent standard curves, thereby improving precision. Samples were assayed for FLm by aliquoting 10 μl into a 1.5 ml centrifuge tube and diluting with 310 μl of 0.1 M NaOH. An aliquot (100 μl in triplicate) was read against an FLm standard curve using a fluorescent plate reader (Fluostar Optima: BMG Labtech – Germany), with the excitation and emission wavelengths set at 485 nm and 520 nm respectively. All samples were diluted to the same FLm concentration (between 50 -100 μM) using SDS buffer.

2.3.3 Reduction

A 50 μl FLm-labeled aliquot was reduced with the addition of 4 μl of SDS buffer containing 50 mM tris (2-carboxyethyl)phosphine (TCEP) (pH 7.0). The sample was vortexed, then incubated for 60 minutes at room temperature in the dark.
2.3.4. *Labelling of oxidised (newly reduced) protein thiols*

After reduction, Texas red maleimide (TRm, Invitrogen) labelling was performed by first diluting the TCEP concentration of the sample with the addition of 50 µl of SDS buffer. Then 5 µl of 5 mM TRm was added, the sample was vortexed briefly, then incubated for 60 minutes at room temperature in the dark. Excess TRm was removed with the addition of 400 µl of ethanol and incubated for at least 60 minutes at -20°C to precipitate proteins. Samples were then centrifuged for five minutes (8000g at 4°C) and the supernatant (and excess dye) was discarded. The sample was re-suspended in 100 µl of SDS buffer and the ethanol rinsing procedure was performed twice more. After the third rinse, the remaining protein pellet was re-suspended in 100 µl of SDS buffer before being assayed for FLm and TRm.

2.3.5 *Fluorescent Assay*

A standard curve for FLm and TRm was constructed by adding 16 µl of 1.5 mM dye to 384 µl ovalbumin solution (20 mg/ml in SDS buffer) and then diluting with SDS buffer. The assay was performed by diluting 10 µl of sample or standard with 310 µl of 0.1 M NaOH, and 100 µl was assayed. Fluorescence was measured using a fluorescent plate reader (Fluostar Optima), (FLm: ex 485 nm, em 520 nm; TRm: ex 595 nm, em 610 nm). The concentrations of FLm and TRm in the samples were calculated from a second order polynomial standard curve. The percentage of oxidised protein thiols was calculated from the equation: TRm concentration / (TRm concentration + FLm concentration) x 100.
2.3.6 Protein assay

The final protein concentration of the sample was determined using a modified BioRad DC protein assay. The working reagent A’ and the sample were diluted two fold with double deionised water. The assay was performed by pipetting 20 μl of sample, 70 μl of reagent A’ and 170 μl of reagent B into a clear, flat bottom 96-well plate. Absorbance was measured at 750 nm and the protein concentration of samples was calculated using linear regression.

2.3.7 Ellman’s Assay

Ellman’s assay was performed using a modified Ellman’s method (Ellman 1959). Crushed muscle samples were subjected to 20% TCA/acetone treatment and solubilisation in SDS buffer as described previously, with the exception that no FLm was added. Samples were first diluted 1/3 with double deionised water before being measured for reduced protein thiols and protein concentration, respectively. For the Ellman’s assay, 100 μl (in triplicate) were aliquoted into a 96-well plate and 100 μl of Ellman’s reagent (DTNB) was added before being incubated at room temperature for 15 minutes, and measured for absorbance at 412 nm. A molar extinction coefficient of 14,150 M⁻¹ cm⁻¹ was used (Riddles et al. 1983; Riener et al. 2002; Eyer et al. 2003). A 20 μl aliquot (in triplicate) was then taken for BioRad DC protein assay, and reduced protein thiol levels were expressed per mg protein.

2.3.8 Gel Electrophoresis

FLm and TRm Gel Standards

In order to quantify reduced and oxidised thiols of specific protein bands using SDS PAGE, in-gel standard curves for FLm and TRm labeled thiols were created from standards
previously described for the 96-well plate assay. FLm and TRm standards were combined (usually in a ratio of three parts FLm standard to one part TRm standard) and further diluted with SDS buffer before a two-fold dilution with gel sample buffer (125 mM Tris pH 6.8, 4% SDS, 30 % (v/v) glycerol, 0.02 % bromophenol blue, 0.2 M dithiothreitol). The final concentration of top standards for FLm and TRm were 1.2 \( \mu \text{M} \) and 0.4 \( \mu \text{M} \) respectively, with 10 \( \mu \text{l} \) applied to the gel.

\textit{Sample Preparation for Gel Electrophoresis}

To ensure that the quantity of labeled protein thiols loaded on the gel corresponded to that of the standard curves, samples were diluted with SDS Buffer to 15 \( \mu \text{M} \) based on the FLm concentration established in the 96-well plate assay. Samples were further diluted two fold with gel sample buffer, heated for five minutes at 95 °C, with 10 \( \mu \text{l} \) applied to the gel (about 1.5 \( \mu \text{g} \) of protein).

\textit{Gel and Buffer Compositions}

Gel electrophoresis was performed using the BioRad Mini Protean III system. Gel and buffer compositions were based on those described previously (Kohn \textit{et al.} 2006), with the exception that a 12% polyacrylamide gel was used, and 5 mM DTT was added to the top running buffer. Electrophoresis was carried out at 8 mAmps with voltage not exceeding 150V for 10 hours at 4 °C, with the buffer in the lower chamber stirred with a magnetic stirrer throughout.
Gel Analysis

Each gel was scanned (Typhoon Trio, GE Health, Australia) for fluorescence (FLm: ex 485 nm, em 520 nm; TRm: ex 595 nm, em 610 nm). The bands were quantified by densitometry using image J version 1.41 software (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA,) using the integrated density function after first removing the background. To assess the level of protein thiol oxidation of specific protein bands, dominant bands were compared against FLm and TRm in-gel standard curves using polynomial regression. To compare the SDS PAGE with the 96-well plate assay, the signal for the whole lane was used for both standards and samples. After gels were scanned, the molecular weight of major bands was calculated by matching band positions with protein molecular weight standards (BioRad, kaleidoscopoe pre-stained molecular weight marker), which were clearly visible at the TRm wavelength.

2.3.9 Statistical Analysis

All data is presented as means ± SEM. Means were compared using a t-test, or one-way ANOVA with repeated measures where appropriate. Significance was accepted at p < 0.05.

2.4 RESULTS

2.4.1 Development of dual labelling technique

The quantitative labelling of tissue samples involves the labelling of reduced and oxidised protein thiols on the same protein extract with different fluorescent dyes. The labeled sample is then assayed using a 96-well plate fluorimeter, with the option of separating
proteins with SDS PAGE and then scanning for fluorescence. The technique incorporates several steps, and the effectiveness of the following steps was evaluated: 1) Protein solubilisation, 2) labelling protein thiol groups with the first fluorescent tag (FLm), 3) removing excess FLm, 4) reducing oxidised protein thiols with TCEP, 5) labelling newly reduced protein thiol groups with the second fluorescent tag (TRm), and 6) removing excess TRm.

1) Protein Extract Solubilisation. Following preliminary studies, we developed an SDS buffer (0.5% SDS and 0.5 M Tris, pH 7.0) to solubilise proteins. The effectiveness of the SDS buffer to solubilise proteins was tested by assaying the amount of residual protein which did not dissolve during the FLm labelling procedure. Residual protein was solubilised in 0.2 M NaOH with heating and vortexing until completely dissolved, then assayed for protein. Of the total protein accounted for, 96.6 ± 0.01% (n = 17) dissolved during the initial solubilisation with FLm labelling. This data indicates the protein solubilisation procedure was effective in muscle tissue proteins.

2) FLm Labelling and 3) Excess Flm Removal. To prevent artefactual oxidation occurring during solubilisation, FLm was added during the protein solubilisation procedure. A concentration of 0.54 mM FLm was sufficient to label protein thiols (Fig. 2.1.1), with labelling complete in 15 minutes (55 ± 1 nmol/mg FLm, n = 4) and stable for at least 60 minutes (55 ± 3 nmol/mg FLm). To further test the completeness of the FLm labelling reaction, tissue extract was labeled with FLm (0.54 mM) for 30 minutes, then immediately incubated with TRm (0.24 mM) for a further 30 minutes. TRm accounted for 0.30 ± 0.01% (n = 4) of the total protein thiol content, indicating that the FLm labelling reaction was
more than 99.7% complete after 30 minutes. In all subsequent experiments, FLm labelling involved incubation for 30 minutes at a final concentration of 0.54 mM.

Non-specific (not associated with the maleimide reactive group) interactions between FLm and non-protein components of tissue extract could lead to an overestimation of protein thiols in tissue. Non-specific interactions were tested by first labelling the tissue extract with TRm to block protein thiols, then immediately labelling with FLm. FLm accounted for only 0.6 ± 0.02% (n = 4) of the total protein thiol content. As an alternative, NEM was used to block protein thiols prior to FLm labelling. FLm accounted for only 0.48 ± 0.16 nmol/mg (or approximately 1%) of the 46.0 ± 0.7 nmol/mg (n = 4) of protein thiols measured in samples not treated with NEM. These data indicate that non-specific interactions of FLm with tissue extract were of little consequence and they also indicate that precipitation was effective in removal of excess FLm. Finally, comparable results were achieved for both ovalbumin (within 2%) and tissue sample (within 6%) when we compared the FLm assay with the Ellman’s assay for measurement of protein thiols (Fig. 2.1.2), indicating consistency between the techniques.

Once protein thiols had reacted with FLm, excess FLm was removed. We chose to remove un-reacted dye with ethanol rather than other methods such as desalting columns, since high levels of protein loss have been reported using such techniques (Riederer et al. 2008). Excess FLm was removed by precipitating proteins with ethanol, centrifuging, and rinsing the resulting pellet again with ethanol. Protein loss during this procedure was minimal, with protein recovery of 96.1 ± 0.04% (n = 9).
To assess the effectiveness of removing excess FLm, the protein sample was re-solubilised in SDS buffer after each (of two) ethanol precipitation(s). There was no significant (p = 0.12) reduction in FLm signal between the first (64.6 ± 1.8 nmol/mg) and second (63.5 ± 0.5 nmol/mg) ethanol precipitation (n = 4). In addition, after each precipitation, excess FLm was also measured in the ethanol supernatant. Of the total excess FLm removed, 98.2 ± 0.01% (n = 4) was accounted for in the ethanol supernatant from the first precipitation. These data indicate that one ethanol precipitation was sufficient to remove excess FLm.

4) Reduction with TCEP. Following FLm removal, oxidised protein thiols were reduced with TCEP prior to being exposed to the second fluorescent dye (TRm). Final concentrations higher than 4 mM TCEP reduced the labelling efficiency of TRm (Fig. 2.2.1). This interference has been noted by others (Burmeister et al. 1999), but could be prevented by a two-fold dilution of the sample with SDS buffer following the reduction reaction and prior to labelling with TRm. Using this protocol, final TCEP concentrations of 2.8 - 5.3 mM were sufficient to reduce the tissue sample without causing interference with the TRm-labelling reaction. For a concentration of 3.8 mM TCEP, the reduction reaction was complete in 30 minutes at room temperature (31.94 ± 0.5 % TRm, n = 4), and the signal was stable for up to 90 minutes (31.9 ± 0.8 % TRm, n = 4). We tested if it was possible to remove TCEP with ethanol precipitation prior to labelling with TRm. Using this procedure, there was a five-fold reduction in TRm labeled thiols relative to labelling with TRm in the presence of TCEP (Fig. 2.2.2). This observation indicated that the removal of the TCEP prior to labelling with TRm was undesirable as artefactual oxidation of protein thiol groups could occur following the removal of the reducing agent.
5) TRm Labelling and 6) TRm Removal. A TRm final concentration of at least 0.24 mM was sufficient to label newly reduced protein thiols generated by TCEP reduction in a solution containing 1 mg/ml protein tissue extract (Fig. 2.3.1). TRm labelling was complete in 15 minutes (13.72 ± 0.1 % TRm, n = 4) and the signal was stable for at least 60 minutes (13.81 ± 0.05 % TRm, n = 4). In order to prevent overestimation of oxidised protein thiols, excess TRm was removed by precipitating proteins with ethanol, then re-solubilising the resulting pellet in SDS buffer. This was repeated three times, and the percentage TRm signal was measured between each solubilisation (Fig. 2.3.2). This data demonstrates that TRm carryover was a potentially confounding factor and that one precipitation was insufficient to remove excess TRm. To further assess TRm carryover, the TRm signal was measured in the ethanol supernatant after each of four consecutive precipitations. Of the total TRm removed, 99.6 ± 0.03% (n = 4) was accounted for in the ethanol of the first and second precipitations. These data indicate that three ethanol precipitation/re-solubilisation repetitions were sufficient to remove excess TRm. Ethanol added in the ratio of two to four times the sample buffer volume was most effective in removing excess TRm (Fig. 2.3.4).

Non-specific binding (not associated with the maleimide reactive group) by TRm to protein, would lead to an overestimation of the level of protein thiol oxidation in tissue. Non-specific interactions were tested by first labelling the tissue extract with 0.54 mM FLm to block protein thiols, then immediately labelling with 0.24 mM TRm. TRm accounted for only 0.3 ± 0.03% (n = 4) of the total signal. Non-specific binding was also tested for by first blocking protein thiols with NEM and then incubating with 0.24 mM TRm. The residual TRm signal was less than 0.2 ± 0.2% (n = 4) of the signal seen in samples not
blocked with NEM. These data indicate non-specific interactions of TRm with tissue extract were negligible and also provided further evidence that the protein precipitation procedure effectively removed excess TRm. Estimated protein thiol concentrations were comparable between Ellman’s assay and TRm assays for both tissue extract (within 7%) and ovalbumin (within 2%), which indicated TRm could be used to accurately measure protein thiol content (Fig. 2.3.3).

*Fluorescent 96-well plate analysis.* Polynomial standard curves were constructed by reacting up to 60 μM FLm and 30 μM TRm with protein thiol groups in an SDS buffer solution containing 20 mg/ml ovalbumin. Excess protein thiol over dye concentration was used to ensure all dye was assayed in its bound (reacted) state. The quantitative accuracy of the dual labelling technique would be compromised if either dye interfered with the excitation or emission signal of the corresponding dye. For dye concentrations up to 60 μM there was no evidence of interference by the corresponding dye (data not shown).

### 2.4.2 Preventing artefactual oxidation during sample preparation

Protein thiols can be oxidised by ambient molecular oxygen (Rossi et al. 2002; Rogers et al. 2006), so artefactual oxidation during protein extraction and solubilisation has the potential to confound results. Acidification has been used to block thiol-disulfide exchange reactions (Rossi et al. 2002; Leichert *et al.* 2008), so we tested the effectiveness of acidification in preventing artefactual oxidation of protein thiols. In the absence of acid, there was a large (almost five fold) increase in the percentage of protein thiol oxidation in samples incubated for 24 hours at room temperature in SDS buffer pH 7.0. In contrast, samples incubated in the presence of TCA/acetone showed a small (2.7%), but significant (p < 0.01) increase in
the percentage of oxidised protein thiols (Fig. 2.4.1). These data established that artefactual oxidation was a possible confounding factor, and that TCA/acetone treatment reduced the oxidation rate.

To gain a better understanding of the rate and extent to which tissue samples are oxidised in 20% TCA/acetone, we examined the extent of protein thiol oxidation over a 15-day period at room temperature. The Ellman’s assay showed a progressive decrease in the levels of reduced protein thiols (Fig. 2.5.1). The dual labelling technique showed both a progressive decrease in reduced protein thiols, and an increase in percentage protein thiol oxidation (Fig. 2.5.2 and 2.5.3). Furthermore, the dual labelling technique showed total protein thiols (reduced + oxidised) progressively decreased over the 15-day period (Fig. 2.5.3), which may be indicative of irreversible thiol oxidation. This data demonstrates, using two different analytical techniques, that TCA/acetone treatment was not completely effective in preventing artefactual oxidation. However, labelling protein thiols shortly after TCA/acetone extraction was sufficient to prevent this artefactual oxidation from occurring.

Having established that artefactual oxidation was a possible confounding factor, and that TCA/acetone treatment reduced the oxidation rate, we then investigated the potential for artefactual oxidation to occur during sample preparation, including the homogenisation (using an Ultra Turrax) and solubilisation steps. We added frozen muscle powder to SDS buffer and homogenised using the Ultra-Turrax. Artefactual oxidation occurred during the homogenisation procedure as there was an increase in protein thiol oxidation from 11.8 ± 0.3% in control (non-homogenised) samples, to 56 ± 7% in samples that were homogenised. We then tested if TCA/acetone treatment prevented artefactual oxidation during the homogenisation procedure. In these samples, frozen muscle powder was first
treated with TCA/acetone. Those that were then homogenised had significantly less (p < 0.05) oxidation (6.6 ± 0.3%) than those that were not homogenised (11.8 ± 0.3%, n = 4). A possible explanation for the difference is that homogenisation increased the exposure of protein thiol groups to TCA and reduced oxidation prior to FLm labelling. Taken together, these data indicate that artefactual oxidation during sample preparation could be most effectively prevented by performing homogenisation in a TCA/acetone medium.

In another experiment using a different muscle sample, we tested the possibility that artefactual oxidation could occur during the protein solubilisation procedure. Protein thiol oxidation was 21 ± 1% (n = 4) when the FLm was added after protein solubilisation, but this decreased to 15.5 ± 1.5% (n = 4) when FLm was added prior to protein solubilisation (Fig. 2.4.2). This data indicated that artefactual oxidation during solubilisation could be prevented by the addition of the first fluorescent tag (FLm) prior to solubilisation.

2.4.3 Application: Quantitative assessment of protein thiols in dystrophic (mdx) muscle
Since oxidative stress has been reported in mdx mice (a model for muscular dystrophy) (Disatnik et al. 1998; Rando et al. 1998), we used the dual labelling technique to assess the oxidation state of protein thiols in the skeletal muscle of both mdx and control (c57) mice. While the Ellman’s assay showed no significant difference (p = 0.56) between control (c57) and mdx mice (Fig. 6.1), the dual labelling technique detected significantly (p < 0.05) more protein thiol oxidation in the muscles of mdx mice (Fig. 6.2).

Labeled proteins could be further separated by SDS PAGE, and this offered the opportunity to examine changes in the level of thiol oxidation of specific protein bands. In order to
quantify TRm and FLm labeled protein thiols, we used an in-gel standard curve utilizing the standards created for the 96-well plate assay (Fig. 7).

The oxidation states of the two most dominant proteins bands were evaluated (Fig. 2.8.1). The most dominant band (band 1) with a molecular weight of approximately 43,000 kDa, presumably rich in actin, showed a similar state of oxidation in both mdx and c57 mice. However, another dominant band (band 2) with a molecular weight of approximately 67,000 kDa, possibly rich in albumin, was significantly more oxidised, particularly in the muscles of mdx mice (Fig. 2.8.2). This data shows that the SDS PAGE stage of the dual labelling technique was sensitive enough to detect changes in the level of thiol oxidation of individual protein bands in biological populations.

Non-protein components of biological samples, such as glutathione, are removed during the TCA/acetone extraction step. However, carryover of fluorescent dye had the potential to interfere with the protein thiol estimates using the 96-well plate method. Non-protein molecules including glutathione and un-reacted dye are removed during gel electrophoresis, so we tested the consistency of protein thiol estimation between the 96-well plate and SDS PAGE assay methods by analysing the oxidation state off all the proteins contained in the entire lane (n = 4). The level of protein thiol oxidation was comparable between the 96-well plate assay and SDS PAGE (Fig. 2.9), indicating non-protein components such as glutathione, and un-reacted dye caused little interference.
**Fig. 2.1:** FLm labelling reaction. (Fig. 2.1.1) FLm labelling reaction during protein solubilisation. A 2 mg/ml muscle protein extract in SDS buffer was labelled with 0.125 – 0.9 mM FLm. (*) indicates significantly different from 0.54 mM FLm value. Nmol/mg refers to soluble protein. (Fig. 2.1.2) Quantitative assessment of protein thiols. Pre-solubilised protein solution and ovalbumin solution (2 mg/ml) were assayed for protein thiols with Ellman’s reagent and FLm. (*) indicates significantly different from Ellman’s value. Nmol/mg refers to soluble protein. (Mean ± SEM, n = 4 for all experiments).
Fig. 2.2: TCEP reduction. An aliquot of 2 mg/ml FLm labeled muscle tissue protein extract solution was reduced with TCEP for 60 minutes, then labelled with 0.24 mM TRm. (Fig. 2.2.1) Effect of TCEP concentration on the effectiveness of the TRm labelling reaction. (*) indicates significantly different from 3.8 mM TCEP value. (Fig. 2.2.2) TRm labelling reaction with and without the removal of TCEP (with ethanol precipitation) prior to TRm labelling. (*) indicates significant difference between groups. (Mean ± SEM, n = 4 for all experiments).
Fig. 2.3: TRm labelling reaction and removal. (Fig. 2.3.1) Effect of TRm concentration on protein thiol labelling. After FLm labelling, TCEP reduction, and a two-fold dilution with SDS buffer, 0.1 - 0.46 mM TRm was used to label 1 mg/ml (reduced) muscle protein extract solution and incubated for 60 minutes. (*) indicates significantly different from 0.24 mM TRm value. (Fig. 2.3.2) Removal of excess TRm with three repeated ethanol precipitations. After FLm labelling and reduction, samples labeled with 0.24 mM TRm, were precipitated, then re-solubilised in SDS buffer three consecutive times and analysed for FLm and TRm after each precipitation. (*) indicates significantly different from 3rd rinse value. (Fig. 2.3.3) Quantitative assessment of protein thiols with TRm and Ellman’s reagent for measuring reduced thiols in 2 mg/ml pre-solubilised muscle tissue protein extract and ovalbumin. Nmol/mg refers to soluble protein. (Fig. 2.3.4) TRm signal strength using different ratios of ethanol to buffer volume to remove unreacted TRm. (*) indicates significantly different from value using Ellman’s reagent. (Mean ± SEM, n = 4 for all experiments).
Fig. 2.4: Prevention of artefactual oxidation during sample preparation. (Fig. 2.4.1) Dual labelling technique showing percentage of oxidised thiols in samples incubated for 0 and 24 hours in either SDS buffer pH 7.0 or 20% TCA/acetone. (*) indicates significantly different from time 0 value. (Fig. 2.4.2) The effect of adding 0.54 mM FLm to 50 μl of 2mg/ml muscle tissue protein extract either before, or after the solubilisation step. (*) indicates significant difference between groups. (Mean ± SEM, n = 4 for all experiments).
Fig. 2.5: TCA/acetone treatment and oxidation by ambient molecular oxygen. (Fig. 2.5.1) Ellman’s assay showing protein thiols (nmol/mg soluble protein) in samples incubated in 20% TCA/acetone over 15 days. (Fig. 2.5.2) Dual labelling technique showing total thiols (nmol/mg soluble protein) in samples incubated in 20% TCA/acetone over 15 days. (Fig. 2.5.3) Dual labelling technique showing the percentage of oxidised thiols in samples incubated in 20% TCA/acetone over 15 days. Nmol/mg refers to soluble protein. (*) indicates significantly different from time 0. n = 4 for all experiments.
Fig. 2.6. Quantitative assessment of protein thiols in dystrophic (mdx) muscle. (Fig. 2.6.1) Ellman’s assay of protein thiols for c57 and mdx muscle tissue. (Fig. 2.6.2) Dual labelling technique showing the percentage of oxidised thiols for c57 and mdx muscle tissue. (*) indicates significantly different from c57. Nmol/mg refers to soluble protein. (Mean ± SEM, n = 4 for all experiments).
Fig. 2.7: SDS PAGE Fluorescent standards. (Fig. 2.7.1) FLm scan of gel standards, and associated FLm gel standard curve working range 0 - 5 pmol. (Fig. 2.7.2) TRm scan of gel standards, and associated TRm gel standard curve working range 0 – 1.25 pmol. Note: replicates were not used for SDS PAGE standards to allow more lanes for samples.
**Fig. 2.8**: SDS PAGE c57 and mdx. (Fig. 2.8.1) TRm scan of samples from c57 mice. (Fig. 2.8.2) Comparison of the oxidation state of major protein bands for the c57 and mdx samples. (*) indicates significant difference between protein bands 1 & 2. (#) indicates significant difference between c57 and mdx. (Mean ± SEM, n = 4).
Fig 2.9. Comparison between 96-well plate assay and SDS PAGE. Mdx and c57 samples were analysed using both the 96-well plate assay and SDS PAGE. For SDS PAGE, total protein in the whole lane was analyzed. (Mean ± SEM, n = 4).
2.5 DISCUSSION

This study describes the development a fluorescent dual labelling technique to quantitatively measure both reduced and oxidised protein thiols in the same tissue sample. This ratio-metric measure is independent of protein concentration and is sensitive enough to detect changes in level of protein thiol oxidation in biological populations. Several challenging problems which have previously limited the accuracy and precision of measuring protein thiols in biological samples such as skeletal muscle were addressed.

The first challenge was to develop a reproducible protein solubilisation method that was compatible with fluorescent dye labelling. Proteins from muscle tissue are difficult to solubilise (Shaw et al. 2003; Thierry 2009). Furthermore, saturation labelling of proteins has been reported to result in considerable loss of protein, with protein recovery reported in the range of 11-50% using a variety of detergents (Riederer et al. 2008). Incomplete solubilisation and recovery of proteins after labelling can lead to: (a) a loss of accuracy if solubilised proteins are not representative of tissue and (b) a loss of precision if the degree of solubilisation of individual proteins varies from sample to sample. Different methods to solubilise proteins from muscle tissue were tested, and the recovery of protein after maleimide labelling and ethanol precipitation was analysed. A combination of physical techniques (homogenisation, sonication and centrifuge) combined with an SDS buffer resulted in excellent protein solubilisation.

A second challenge was to prevent artefactual oxidation. Results showed there was a potential for artefactual oxidation to occur at several steps in the measurement procedure. Acidification was used during sample preparation both to decrease thiol/disulfide exchange
reactions, as well as destroy cellular reductants such as NADPH and NADH (Passonneau and Lowry 1993). Labelling free protein thiols with FLm during the solubilisation procedure was important to prevent oxidation from occurring during this critical step. The addition of TRm in the presence of the reducing agent was required to prevent oxidation of newly reduced protein thiols that occurred if the reductant was removed prior to labelling. Since the reductant (TCEP) interfered with the TRm reaction (Burmeister et al. 1999), it was necessary to first dilute the TCEP concentration with SDS buffer before adding TRm. By adopting these strategies, the potential for artefactual oxidation during sample preparation and measurement was significantly reduced.

Another issue, specific to protein thiol measurement, is that some thiol groups may be readily accessed by thiol reactive probes, while other thiols may be inaccessible due to the three dimensional folded state of proteins. In order to overcome this, and to ensure that all protein thiols were captured during measurement, protein denaturing conditions during both the extraction (using TCA/acetone) and the labelling (using SDS buffer) procedures were used. However, it has been shown that protein thiols differ in their susceptibility to oxidation (Winterbourn and Hampton 2008), and surface proteins may be more likely to be exposed to oxidative stress in vivo, and therefore more likely targets of redox signaling. It could be argued that by capturing the entire protein thiol pool, the significance of the signal generated by redox sensitive thiol groups may be diluted. While more research on the specific proteins involved in redox signaling is required, the techniques application has been demonstrated to be effective and shown it to be sufficiently sensitive to detect changes in level of protein thiol oxidation of muscle tissue in biological populations exposed to oxidative stress.
Various techniques for the measurement of thiols in proteins have been previously described and include thiol-labelling agents such as maleimide, iodoacetate, iodoacetamide, and thiosulfates (Rossi et al. 2002; Eaton 2006). The most simple and a common approach involves measuring the reduced state of protein thiols using techniques such as the Ellman’s assay (Freeman and Meredith 1989; Balcerzyk et al. 2003; Prakash et al. 2004; Varsanyi et al. 2004; Eaton 2006; Hawkins et al. 2009). However, measuring both reduced and oxidised protein thiols provides more information regarding the effect of oxidative stress. For example, the chronic effect of the oxidative stress condition on total protein thiols (reduced plus oxidised) can be gauged and the relative degree of protein thiol oxidation can be compared between different experimental conditions. Furthermore, measuring both reduced and oxidised protein thiols improves the sensitivity in detecting changes in protein thiol oxidation. As this study and others have shown, proteins in tissue samples exist in a predominantly reduced state (Paget et al. 2003). Consequently, small changes in level of protein thiol oxidation can be difficult to detect using techniques which only measure reduced protein thiols, because they are measured against a substantial background signal of reduced thiols. This was evident in the data. The Ellman’s assay of reduced protein thiols showed no significant difference between mdx and c57 mice. However, a significant difference in level of protein thiol oxidation was observed when using the ratio-metric dual labelling technique.

The dual labelling technique has been designed to be a first pass method to identify oxidative stress conditions leading to protein thiol oxidation in biological tissues such as muscle. For many proteins, thiol oxidation has been suggested to affect their function in
both health and disease (Paget et al. 2003; Eaton 2006; Winterbourn and Hampton 2008). Consequently, it is of interest to identify such proteins, and one approach is to use gel electrophoresis. The dual labelling has been demonstrated to be compatible with gel electrophoresis and changes in the oxidation state of individual protein bands can be detected and quantified. As proteins can co-migrate with one-dimensional SDS PAGE, further separation of proteins using two-dimensional gel electrophoresis would be desirable to identify specific proteins by mass spectrometry. The SDS buffer we used is not compatible with two-dimensional gel electrophoresis. However, it should be possible to extend the dual labelling technique to two-dimensional gel electrophoresis by solubilising the final dual labeled protein pellet in an appropriate buffer (Riederer et al. 2007; Lui et al. 2010).

2.6 ACKNOWLEDGEMENTS

This research was supported by an Australian Research Council Linkage Grant and grants from the National Health and Medical Research Council of Australia. The mdx and c57 mouse tissue was provided by Jessica Terrill, Department of Anatomy and Human Biology (University of Western Australia). The assistance of Ralph Zerbes for the experiments related to Fig 2.5 was greatly appreciated.
CHAPTER 3

THE EFFECT OF EXERCISE AND RECOVERY ON THE LEVEL OF PROTEIN THIOL OXIDATION OF RAT SKELETAL MUSCLES
ABSTRACT

The effect of exercise and recovery on the level of thiol oxidation of muscle proteins in vivo has yet to be investigated. The goal of this study was to examine the effect of both three-minutes high intensity swimming to fatigue and 30 minutes of moderate intensity non-fatiguing swimming on the level of thiol oxidation of muscle proteins in overnight fasted male Wistar rats. With the help of a fluorescent dual labelling technique to measure both reduced and oxidised protein thiols in skeletal muscles, the response of the level of protein thiol oxidation of muscle proteins to exercise was shown to be muscle fibre specific. In response to both moderate intensity and high intensity swimming to fatigue, there was a significant (P < 0.05) increase in the level of thiol oxidation of total proteins in the red gastrocnemius muscle. In contrast, these two exercise protocols had no effect on the level of protein thiol oxidation of the white gastrocnemius muscle, and resulted in a fall in the level of thiol oxidation of total proteins in the soleus muscle. Both exercise protocols had no effect on protein carbonyl levels in the muscles examined. SDS PAGE revealed an increase in level of protein thiol oxidation of the myosin and glycogen phosphorylase protein bands in the red gastrocnemius muscle in response to high intensity swimming, while the other proteins examined remained unaffected. During the recovery period following high intensity exercise, the level of protein thiol oxidation in the red gastrocnemius muscle increased transiently, peaking at 15 minutes before slowly declining over the following 45 minutes of recovery. In conclusion, both moderate intensity exercise and high intensity exercise to fatigue resulted in an exercise and fiber-specific change in the level of protein thiol oxidation of muscle proteins.
3.1 INTRODUCTION

During muscle contraction, there is a marked accumulation of reactive oxygen/nitrogen species (RONS) in skeletal muscles (Davies et al. 1982; Finaud et al. 2006; Reid 2008; Fisher-Wellman and Bloomer 2009). There is evidence that the resulting increase in oxidative/nitrosative stress plays important physiological roles such as that of mediating muscle fatigue and adaptive responses to exercise and disuse (Barclay and Hansel 1991; Allen and Tresini 2000; Bruton et al. 2008; Lamb and Westerblad 2011; Reid and Moylan 2011). This is supported, in part, by studies that show antioxidants, such as dithiothreitol or N-acetylcysteine (NAC) attenuate muscle gene expression post-exercise (Pinkus et al. 1996; Allen and Tresini 2000; Murphy et al. 2008; Petersen et al. 2012) as well as muscle fatigue in vitro and in vivo (Supinski et al. 1997; McKenna et al. 2006) in both rodent (Shindoh et al. 1990) and human muscle (Reid et al. 1994; Travaline et al. 1997; Medved et al. 2004; McKenna et al. 2006; Bentley et al. 2012), whereas oxidants such as hydrogen peroxide increase fatigue (Andrade et al. 1998; Reid 2008; Lamb and Westerblad 2011).

During strenuous physical exercise, increased RONS levels can cause a range of oxidative modifications including the irreversible damage to macromolecules such as DNA, lipid membranes and proteins (Sumida et al. 1989; Duthie et al. 1990; Barreiro et al. 2005) and other modifications which are reversible such as the oxidation/nitrosylation of the thiols (or sulfhydryl) residues of the amino acid cysteine in muscle proteins (Eaton 2006; Winterbourn and Hampton 2008). The reversible formation of these bonds can affect protein function, therefore enabling RONS to act as a signaling mechanism affecting a wide range of cellular functions (Ferreira et al. 2008).
Studies performed with isolated muscle proteins indicate that a number of proteins are susceptible to reversible thiol oxidation, including the calcium release channels, the proteins associated with the myofilaments (myosin, actin, troponin and tropomyosin) (Dalle-Donne et al. 2003; Dalle-Donne et al. 2003; Prochniewicz et al. 2008; Mollica et al. 2012), several enzymes such as glycogen phosphorylase (Cappel and Gilbert 1986), and redox sensitive kinases or phosphatases to list a few (Allen et al. 2008; Ferreira et al. 2008). What is still unclear is the unanswered question of whether the level of thiol oxidation of these and other muscle proteins is affected by exercise and recovery from exercise. The closest study to provide an answer to this question was performed *in situ* using electrically stimulated extensor digitorum longus muscle fibres where free thiols were reported to fall 15 minutes into the recovery period and returned to resting levels by 60 minutes of recovery (McArdle et al. 2001). However, oxidised protein thiols and individual proteins were not examined.

The lack of information regarding the response of muscle protein thiol oxidation to exercise *in vivo* is due, in part, to the absence of a method sensitive enough to allow the detection of small differences in the level of thiol oxidation of muscle proteins, as well as various technical issues in their measurement (Jones 2006; Fisher-Wellman and Bloomer 2009; Palomero and Jackson 2010). Recently, however, we have published a new technique that allows for the direct quantification of both reduced and oxidised protein thiols of muscle extracts (Chapter two (Armstrong et al. 2011)). This technique can also be extended, using one dimensional SDS PAGE, to measure the level of thiol oxidation of specific major proteins bands (e.g. myosin, actin, troponin, glycogen phosphorylase). Given that the
oxidative stress of skeletal muscle increases with contraction, the primary aim of this study was to examine the effect of both a fatiguing and non-fatiguing exercise on the level of thiol oxidation of muscle proteins to determine whether this process is affected by the pattern of muscle activity and muscle fibre composition.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Unless otherwise stated, all chemicals and reagents were obtained from Sigma Aldrich (Castle Hill, Australia). Ethanol (99.5%) and acetone (99.5%) were purchased from Redox chemicals, and double deionised water was used throughout. Protein molecular weight standards were purchased from BioRad, Australia. BODIPY FL-N-(2-aminoethyl) maleimide (FLm) and texas red maleimide (TRm) were purchase from Invitrogen, Australia.

3.2.2 Animals

Male Wistar rats were purchased from the Animal Resources Centre, Western Australia. All animals were fed ad libitum and housed in the pre-clinical animal facility and all procedures were conducted in accordance with the guidelines of the University of Western Australia and the National Health and Medical Research Council of Australia Animal Ethics. Rats were fasted for 24 hrs prior to testing.
3.2.3 Exercise protocol

Rats were randomly assigned into six groups of six animals, with each group except one subjected to the following exercise and recovery protocol. Five of these groups were subjected to a rest or three minutes of high intensity exercise to fatigue followed by either 0, 15, 40 or 60 minutes of recovery. Another group of animals was subjected to 30 minutes of moderate intensity exercise with no recovery period. The rats subjected to high intensity exercise to fatigue were forced to swim for three minutes in water preheated at 32°C as described in detail by Ferreira and colleagues (2001), with a lead weight equivalent to 10% body mass attached to the base of their tails and with one third of the weight removed in succession as the animal tires. In contrast, all animals subjected to moderate intensity exercise swam with a lead weight equivalent to 0.5% of body mass attached to the tail, a protocol chosen on the grounds that this type of exercise can be sustained for in excess of one hour by these animals (Raja et al. 2003).

3.2.4 Muscle sampling

Prior to tissue sampling, rats were anesthetized with isoflurane (4% isoflurane/96% oxygen; Bomac, Australia) and were maintained under general anesthesia while the soleus and the red and white portions of the gastrocnemius muscle were collected. These muscle samples were chosen on the grounds that the soleus, red gastrocnemius and white gastrocnemius muscles are rich in type I, Ila and IIb fibres, respectively (Armstrong and Phelps 1984). Immediately after sampling, each muscle sample was freeze-clamped between aluminum clamps pre-cooled in liquid nitrogen before being stored at -80°C until analysis.
3.2.5 Measurement of reduced and oxidised protein thiols

Reduced and oxidised protein thiols were measured as described previously (chapter two) (Armstrong et al. 2011). Briefly, weighed muscle samples were crushed with a pestle and mortar submerged in liquid nitrogen. Samples were then acidified in a 20% (w/v) trichloroacetic (TCA) in acetone solution to quench the oxidised thiols. TCA was subsequently removed with an acetone wash, and the protein extract was solubilised while labeled with the fluorescent tag (FLm). Excess FLm was removed with ethanol precipitation before reduction of the extract was performed using tris (2-carboxyethyl) phosphine (TCEP). Then, labelling with the second fluorescent tag (TRm) was performed in the presence of TCEP, with excess TRm subsequently removed with a series of three ethanol washes. The labeled samples were re-solubilised in SDS/Tris buffer, after which, fluorescence was measured using a fluorescent plate reader (Fluostar Optima; FLm: ex 485 nm, em 520 nm; TRm: ex 595 nm, em 610 nm). Standard curves for FLm and TRm were constructed by adding a known quantity of fluorescent dye to an ovalbumin solution. The concentrations of FLm and TRm in the samples were calculated from a second order polynomial standard curve. The percentage of oxidised protein thiols was calculated from the equation: TRm concentration / (TRm concentration + FLm concentration) x 100. Protein concentration (mg/ml) was determined as described previously (section 2.3.6).

3.2.6 Measurement of protein carbonylation

Protein carbonyl content was determined by exposing each muscle extract to 2,4-dinitrophenylhydrazine (DNPH) as described previously (Levine et al. 1990; Hawkins et al. 2009). Briefly, muscles were crushed with a pestle and mortar submerged in liquid nitrogen, and homogenized in 20% (w/v) TCA in acetone. Then, each sample was
centrifuged and the supernatant removed, leaving the protein pellet undisturbed. The pellets were re-suspended in 10 mM DNPH in 2 M HCl and incubated for 30 minutes at room temperature. Excess dye was removed by washing the samples three times with ethyl acetate/ethanol (1:1) before being dissolved in 6 M guanidine hydrochloride. Absorbance was measured spectrophotometrically at 370 nm. Protein concentration (mg/ml) was determined as described previously (section 2.3.6). Carbonyl concentrations were expressed as nmol of carbonyl per mg soluble protein.

3.2.7 SDS PAGE

Prior to SDS PAGE, dual labeled protein samples prepared as described above were diluted to 0.5 mg/ml in SDS/Tris buffer, before being diluted by half with gel sample buffer (125 mM Tris pH 6.8, 4% SDS, 30 % (v/v) glycerol, 0.02 % bromophenol blue, 0.2 M dithiothreitol). Then, 10 µl (2.5 µg protein) was applied to the gel. Gel electrophoresis was performed at 4°C using the high voltage flat bed scanner (Typhoon Trio, GE Health, Australia) and 12.5% precast gels (The Gel Company, USA). The running buffer contained 0.05 M Tris, 75 mM glycine, 0.05% SDS, 5 mM DTT. Electrophoresis was carried out at 250V (not exceeding 20 mAmps) for 1 hr before the voltage was increased to 500V (not exceeding 30 mAmps) for another 6 hours.

3.2.8 Gel analysis

Each gel was scanned for fluorescence (FLm: ex 485 nm, em 520 nm: TRm: ex 595 nm, em 610 nm). The bands were quantified by densitometry using Image J version 1.41 software (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA) using the integrated density function after performing the standard subtract
background function. FLm and TRm scanned images were converted to RGB format and merged into two separate channels. To assess the level of protein thiol oxidation of specific protein bands, dominant bands were enclosed with a box, then the signal from the FLm and TRm channel was taken from exactly the same portion of the protein band by alternating between channels. A percentage measure was calculated by using the equation (TRm signal/(TRm + FLm signal) x 100). In order to account for inter-gel variations between FLm and TRm fluorescent scans, a gel standard was run on each gel. To ensure standards and samples were of a similar relative intensity after fluorescent scanning, FLm and TRm standards from the 96 well plate assay described above, were mixed in a ratio of one part TRm standard to four parts FLm standard. An adjustment factor for each gel was calculated based on the relative intensity of the FLm and TRm signals seen from the scans. After gels were scanned, the molecular weight of major bands was calculated by matching band positions with protein molecular weight standards (BioRad, kaleidoscopoe pre-stained molecular weight marker), which were clearly visible at the TRm wavelength.

3.2.9 Mass spectrometry

Mass spectrometry was used to identify major protein bands. Gels were fixed in 50% methanol, 10% TCA (w/v), rinsed three times in double deionised water before blue silver staining in 10% phosphoric acid, 10% (w/v) ammonium sulphate, 0.12% (w/v) brilliant blue G 250 20% methanol overnight. Then gels were rinsed three times in double deionised water for a total of at least three hours. Gel bands were excised for in-gel digestion, and cut into 1 mm cubes. Gel pieces were destained three times with 100 μL 25 mmM ammonium bicarbonate in 50% (w/v) acetonitrile at 37 °C for 45 minutes. Gel pieces were then dried by vacuum centrifugation. Proteins were digested by the addition of 125 ng
Trypsin in 10 μL of 25 mM ammonium bicarbonate. The digestion reaction proceeded at 37 °C overnight. The digested proteins were extracted by two additions of 20 μL 1% trifluoroacetic acid (TFA) in acetonitrile (ACN) and incubation at room temperature for 20 minutes. Extracts were pooled and dessicated by vacuum centrifugation before being reconstituted in 10 μL 80% ACN, 0.1% TFA. Of the extracts, 0.6 μL was combined with 0.6 μL matrix solution (5mg/mL α-cyano-4- hydroxysuccinamic acid, 10mM Ammonium Citrate, 80%ACN, 0.1%TFA) on a MALDI-TOF plate, and allowed to air dry. Analysis was performed with a 4800 MALDI-TOF/TOF Mass Spectrometer (Applied Biosystems, MA). Parent mass peaks (mass range m/z 800-3000 from combined MS and MS/MS spectra) were submitted to the MASCOT database for identification of peptides, using the following search conditions: Swissprot database, all mammalian species, trypsin digest with allowance for up to one missed cleavage per peptide, no fixed modifications, variable modification of oxidation on methionine residues, MS tolerance of 0.2 Da, MS/MS tolerance of 0.6 Da. The most matched ion scores were conceded based on MS/MS results.

### 3.2.10 Statistical analysis

All data is presented as means ± SEM. Means were compared using a t-test or one-way ANOVA with repeated measures where appropriate, with post hoc analyses performed using a Dunett test. Significance was accepted at p < 0.05.

### 3.3 RESULTS

#### 3.3.1 Effect of exercise on the level of thiol oxidation of muscle protein

In response to both high intensity and moderate intensity swimming, the level of thiol oxidation of total proteins in the red *gastrocnemius* muscles increased by 20% and 25%,
respectively (Fig. 3.1.1). The levels of total protein thiols (reduced plus oxidised) were not affected by either exercise protocol (Fig. 3.1.2), indicating the increase in the proportion of oxidised protein thiols was likely a consequence of reversible protein thiol oxidation. There were no detectable changes in protein carbonyl levels, a measure of irreversible protein oxidation (Fig. 3.1.3).

The level of thiol oxidation of myosin, actin, troponin and glycogen phosphorylase protein bands were readily quantifiable using 1D SDS PAGE and identified using mass spectrometry. In the red gastrocnemius, the level of thiol oxidation of myosin and glycogen phosphorylase increased 15% and 8.7% respectively after high intensity exercise, but no change was seen for the other proteins in response to either exercise protocol (Fig. 3.2).

High intensity and moderate intensity exercise did not result in any changes in the level of protein thiol oxidation, total protein thiols or protein carbonyl levels in the white gastrocnemius muscle (Fig. 3.3). Consistent with these findings, both types of exercise did not affect the level of thiol oxidation of myosin, actin, troponin, glycogen phosphorylase and in this muscle (Fig. 3.4).

In the soleus muscle, the level of total protein thiol oxidation decreased by 24% in response to high intensity exercise, but was not significantly affected by moderate intensity exercise (p = 0.075) (Fig. 3.5.1). Both total protein thiols (Fig. 3.5.2) and oxidised protein thiols (Fig. 3.5.3) decreased in response to both exercise protocols. The level of thiol oxidation of myosin fell by 9% and 18% in response to high and moderate intensity exercise respectively (Fig. 3.6). The level of protein thiol oxidation of troponin decreased in
response to high intensity exercise, but increased in response to moderate exercise (Fig. 3.6), whereas none of the other proteins were affected by either exercise protocol.

### 3.3.2 The level of protein thiol oxidation of the red gastrocnemius muscle during the recovery period following high intensity exercise

Since the largest change in the level of protein thiol oxidation occurred in the red *gastrocnemius* muscle, the pattern of recovery in the level of protein thiol oxidation of this muscle was examined. Against expectation, total protein thiol oxidation levels peaked at 15 minutes post-exercise, with levels 76% higher than those immediately after exercise and were still elevated at 60 minutes post-high intensity exercise (Fig. 3.7.1). The level of protein thiol oxidation in total protein was significantly higher at all time points in recovery, when compared back to the pre-exercise values. In contrast, there was no changes in total protein thiols (Fig. 3.7.2) and protein carbonyl levels (*P* = 0.129, Fig. 3.7.3) during recovery from exercise. The post-exercise increase in the level of protein thiol oxidation was accompanied by a rise in the level of protein thiol oxidation of myosin, actin, glycogen phosphorylase which reached peak levels 15 minutes into the recovery period (Fig. 3.8.1 & Fig. 3.8.2). In contrast, the level of thiol oxidation of troponin decreased 15 minutes into recovery (Fig. 3.8.3). Within 60 minutes of recovery, the level of thiol oxidation of myosin and glycogen phosphorylase were no longer elevated relative to those immediately post-exercise, but actin was still more oxidised and troponin was still more reduced. Compared to pre-exercise values, the level of protein thiol oxidation of myosin and actin were higher at all time points in recovery, that of glycogen phosphorylase was higher at 0 and 15 minutes into recovery, and that of troponin was lower at all time points examined in the recovery period.
**Fig. 3.1:** The effect of resting, three minutes of high intensity exercise to fatigue (High) or 30 minutes of moderate intensity exercise (Mod) on the percentage of protein thiol oxidised (Fig.3.1.1), total protein thiols (reduced plus oxidised) (Fig. 3.1.2), and carbonyl levels (Fig.3.1.3) in the red *gastrocnemius* muscle. Nmol/mg refers to soluble protein. (*) indicates significantly different from resting value. (Mean ± SEM, n = 6 for all groups).
**Fig. 3.2.** The effect of resting, three minutes of high intensity exercise to fatigue (High) or 30 minutes of moderate intensity exercise (Mod), on the percentage of thiol oxidised myosin, actin, troponin and glycogen phosphorylase rich protein bands in the red *gastrocnemius* muscle. (*) indicates significantly different from resting value. (Mean ± SEM, n = 6 for all groups).
Fig. 3.3: The effect of resting, three minutes of high intensity exercise to fatigue (High) or 30 minutes of moderate intensity exercise (Mod) on the percentage of protein thiol oxidised (Fig. 3.3.1), total protein thiols (reduced plus oxidised) (Fig. 3.3.2) and carbonyl levels (Fig. 3.3.3) in the white \textit{gastrocnemius} muscle. Nmol/mg refers to soluble protein. (*) indicates significantly different from resting value. (Mean ± SEM, n = 6 for all groups).
Fig. 3.4. The effect of resting, three minutes of high intensity exercise to fatigue (High) or 30 minutes of moderate intensity exercise (Mod), on the percentage of thiol oxidised myosin, actin, troponin and glycogen phosphorylase rich protein bands in the white gastrocnemius muscle. (*) indicates significantly different from resting value. (Mean ± SEM, n = 6 for all groups).
**Fig. 3.5:** The effect of resting, three minutes of high intensity exercise to fatigue (High) or 30 minutes of moderate intensity exercise (Mod) on the percentage of protein thiol oxidised (Fig. 3.5.1), total protein thiols (reduced plus oxidised) (Fig. 3.5.2), and reduced and oxidised protein thiols (Fig. 3.5.3) in the *soleus* muscle. Nmol/mg refers to soluble protein. (*) indicates significantly different from resting value. (Mean ± SEM, n = 6 for all groups).

Carbonyl data was lost due to technical problems.
Fig. 3.6. The effect of resting, three minutes of high intensity exercise to fatigue (High) or 30 minutes of moderate intensity exercise (Mod), on the percentage of thiol oxidised myosin, actin, troponin and glycogen phosphorylase rich protein bands in the soleus muscle. (*) indicates significantly different from resting value. (Mean ± SEM, n = 6 for all groups).
**Fig. 3.7:** The effect of 0, 15, 30 and 60 minutes of recovery following three minutes of high intensity exercise on the percentage of protein thiol oxidised (Fig. 3.7.1), total protein thiols (reduced plus oxidised) (Fig. 3.7.2), and carbonyl levels (Fig. 3.7.3) in the red *gastrocnemius* muscle. Nmol/mg refers to soluble protein. (*) indicates significantly different from immediate post-exercise (0) value. (Mean ± SEM, n = 6 for all groups).
Fig. 3.8: The effect of 0, 15, 40 and 60 minutes of recovery following 3 minutes of high intensity exercise on the percentage of protein thiol oxidised myosin (Fig. 3.1.1), actin, (Fig. 3.8.2) troponin (Fig. 3.8.3) and glycogen phosphorylase (Fig. 3.8.4) rich protein bands from the red *gastrocnemius* muscle. (*) indicates significantly different from immediate post exercise (0) value. (Mean ± SEM, n = 6 for all groups).
**Fig. 3.9:** SDS PAGE separation of major protein bands after dual labelling procedure applied to red *gastrocnemius* muscle tissue protein extract. Gel scanned at FLm and TRm wavelengths. See methods section of text.


3.4 DISCUSSION

The effect of exercise and recovery on the level of thiol oxidation of muscle proteins in vivo has not previously been investigated. To the best of my knowledge, this is the first study to address this issue and to show that the thiol oxidation of muscle proteins is affected by exercise, but in a manner that is muscle fibre and exercise specific. In addition, SDS PAGE analysis of specific protein bands reveals that the pattern of response of the thiol oxidation of individual proteins is protein-specific, indicating that changes in the level of protein thiol oxidation in response to exercise are a complex and highly specific phenomenon. Finally, this study also shows that the level of protein thiol oxidation in the red gastrocnemius muscle experiences a transient increase during the post-exercise recovery period instead of returning progressively to resting levels as may be expected.

Both the gastrocnemius red and white muscles of rodents are heavily recruited during swimming exercise (Armstrong and Laughlin 1983; Ferreira et al. 2001). The level of protein thiol oxidation in the red gastrocnemius muscle increased in response to both high intensity exercise to fatigue and moderate intensity exercise. These findings are consistent with those of others that have shown that exercise is associated with increased oxidative stress (McArdle and Jackson 2000; Bloomer and Goldfarb 2004; Bloomer et al. 2005; Powers and Jackson 2008; Reid 2008; Fisher-Wellman and Bloomer 2009), and also corroborate for the first time the prediction made by others that exercise in vivo causes an increase in protein thiol oxidation in skeletal muscles as a consequence of increased RONS generation (Reid 1998; Ferreira et al. 2008; Reid 2008). Although others have shown that muscle contraction causes a decrease in reduced protein thiols (McArdle et al. 2001), their findings were obtained using in situ muscle preparations rather being performed in vivo.
Moreover, it is important to stress that a loss of free thiols does not necessarily imply an increase in the level of protein thiol oxidation, since a loss of free thiols does not exclude the possibility that this might result from a number of other oxidative modifications or a fall in total protein thiols as discussed in Chapter two, a limitation which is not shared by the current study.

Although the rise in the level of protein thiol oxidation in the red *gastrocnemius* muscle is consistent with the view that exercise increases oxidative stress, this study clearly shows that the response of this muscle is not typical of all skeletal muscles since the patterns of change in the level of protein thiol oxidation is shown here to be affected by muscle fibre composition. Indeed, both moderate intensity non-fatiguing exercise and high intensity exercise to fatigue resulted in no change in the level of protein thiol oxidation in white *gastrocnemius* muscle and a decrease in protein thiol oxidation level in the *soleus* muscle. Why protein thiol oxidation did not increase in both the white *gastrocnemius* and *soleus* muscles remains to be explained. It is possible that the absence of any increase in protein thiol oxidation level in the white *gastrocnemius* muscle is related to its poor oxidative capacity and associated decreased capacity to generate RONS due to its predominance of type IIb fibers. With respect to the *soleus* muscle, it is possible that the absence of increase in protein thiol oxidation level is related to the preferential recruitment of the red and white *gastrocnemius* muscles compared to the *soleus* muscle during intense swimming as suggested by the absence of glycogen mobilisation in the *soleus* muscle under these conditions (Armstrong and Laughlin 1983; Ferreira *et al.* 2001).
The increase in the level of total protein thiol oxidation as well as that of myosin and glycogen phosphorylase in the red *gastrocnemius* muscle may be associated with the development of muscle fatigue in response to high intensity swimming. Indeed, the functions of several major proteins associated with contraction are sensitive to thiol oxidation (Perkins *et al.* 1997) and may be involved in muscle fatigue. In particular, the thiol oxidation of myosin ATPase inhibits its activity (Yamashita *et al.* 1964; Takamori *et al.* 1976; Tiago *et al.* 2004) and acto/myosin interaction (Diaz *et al.* 1994; Ooizumi and Xiong 2004; Moopanar and Allen 2005; Cooke and Cooke 2007; Murphy *et al.* 2008). Similarly, glycogen phosphorylase has been shown to be thiol redox sensitive and this may influence its activity (Cappel and Gilbert 1986). Given that the level of thiol oxidation of these two proteins increased in response to high intensity swimming to fatigue, it is possible that these changes contribute to the development of muscle fatigue. This interpretation is further supported by the observation that sustained exercise of moderate intensity did not cause a significant increase in the level of thiol oxidation of these two proteins.

It is important to stress that although the results with the red *gastrocnemius* muscle might be taken as evidence that changes in the level of thiol oxidation of proteins may contribute to the fatigue associated with high intensity swimming, in many respects the findings show otherwise. Indeed, high intensity exercise to fatigue and moderate intensity non-fatiguing exercise resulted in a similar increase in total protein oxidation level in the red *gastrocnemius* muscle. Moreover, the relative increase in the level of thiol oxidation of myosin and glycogen phosphorylase was relatively small. Also, the oxidation state of myosin after high intensity swimming to fatigue did not differ from non-fatiguing
swimming, and the level of thiol oxidation of glycogen phosphorylase experienced a similar but not significant increase in response to non-fatiguing compared to fatiguing swimming. With respect to the white *gastrocnemius* muscle, the absence of any increase in the level of thiol oxidation of total as well as the specific proteins examined here does not support the notion that RONS-mediated thiol oxidation of proteins contributes to muscle fatigue. Regarding the *soleus* muscle, the evidence that this muscle is not preferentially recruited during intense swimming implies that fatigue is unlikely to have occurred in this muscle, thus making it difficult to evaluate the role that protein thiol oxidation may play as a mediator of fatigue in this muscle.

One unexpected finding with this study was the marked increase in the level of thiol oxidation of total protein and some but not all of the specific proteins examined here in the red *gastrocnemius* muscle during the recovery period following high intensity exercise to fatigue. This finding is important as it raises further doubt as to the importance of thiol-oxidation as a mediator of fatigue, since recovery would be expected to be associated with a fall, rather than a transient increase, in protein thiol oxidation level. The physiological importance of this pattern of change in the level of protein thiol oxidation remains to be determined. It is possible that since oxidative stress has been shown to increase gene expression (Sen 1998; Xie et al. 1999; Ji 2007; Gomez-Cabrera et al. 2008; Murphy et al. 2008; Powers et al. 2010), the post-exercise rise in intramuscular oxidative stress as reflected by the rise in protein thiol oxidation level may act to stimulate gene expression, thus contributing to the adaptive changes that take place after exercise.
The post-exercise rise in the level of protein thiol oxidation raises the issue of the mechanisms involved. Since RONS generation by mitochondria is sensitive to oxygen concentration (Kowaltowski et al. 2009) and that mitochondria in state III produces considerably less RONS than state IV, it is possible that the return of the mitochondria from state III to state IV during recovery results in an increase in RONS production by mitochondria (Di Meo and Venditti 2001; Jackson 2007). Another possibility relates to the observation that aerobic exercise at intensities above maximum oxygen uptake can cause hypoxia in skeletal muscles. The reperfusion that occurs during recovery triggers the conversion of xanthine dehydrogenase to xanthine oxidase, an enzyme that catalyses the production of RONS (Hellsten et al. 1996; Vina et al. 2000; Volek et al. 2002). It is therefore possible that recovery was accompanied by an increase in the activity of xanthine oxidase which in turn resulted in a RONS-mediated oxidation of protein thiol groups. That this mechanism might play a role is supported by the findings that the levels of free radical markers of oxidative stress are reduced when allopurinal (a xanthine oxidase inhibitor) is administered in both exercising animals and humans (Gomez-Cabrera et al. 2003; Gomez-Cabrera et al. 2005)

In conclusion, this study shows that the level of thiol oxidation of muscle proteins is affected by exercise in a manner that is muscle fibre specific, and that the pattern of response of the thiol oxidation of individual proteins is protein-specific, thus indicating that changes in the level of protein thiol oxidation in response to exercise are a complex and highly specific phenomenon. Finally, this study challenges the notion that increases in the level of protein thiol oxidation may be a significant mediator of muscle fatigue.
3.5 ACKNOWLEDGEMENTS

This research was supported by an Australian Research Council Linkage Grant. Mass spectrometry was performed by Amber Boyatzis.
CHAPTER 4

THE EFFECT OF A MAXIMAL CYCLING EFFORT AND RECOVERY ON THE LEVEL OF THIOL OXIDATION OF MUSCLE PROTEINS IN HUMANS
ABSTRACT

The effect of exercise on the level of thiol oxidation of muscle proteins has never been examined before in humans. For this reason, the purpose of this study was to determine whether the level of thiol oxidation of muscle proteins increases during a maximal sprint effort. Following a familiarisation session, five healthy male athletes aged between 18 and 35 years old were required to perform a 30-second maximal sprint effort on a cycle ergometer following an overnight fast. Blood samples, muscle oxygenation level, and muscle biopsies from the vastus lateralis were performed before and at 0, 15 and 40 minutes into the recovery period. In response to sprinting, there was a significant 40% fall in muscle power together with a 47% decrease in muscle oxygenation level. Labelling of reduced and oxidised protein thiols revealed a 37% decrease in the level of thiol oxidation of total proteins. 1D SDS PAGE was used to quantify the level of thiol oxidation of major protein bands. Following the maximal sprint, there was a 39% decrease in myosin oxidation, a 7.3% decrease in the thiol oxidation state of glycogen phosphorylase, and no significant change in actin. During the 40 minute recovery period, the level of thiol oxidation of total muscle proteins as well as muscle oxygenation increased and remained elevated. In conclusion, these results show that sprinting causes a transient decrease in the level of thiol oxidation of muscle proteins followed by a post-exercise increase above resting levels. These patterns of change during and after exercise may be involved in muscle fatigue and muscle adaptation to training, respectively.
4.1 INTRODUCTION

Intense physical activity is well documented to cause an increase in oxidative/nitrosative stress due in part to the marked accumulation of reactive oxygen/nitrogen species (RONS) in skeletal muscles (Bejma and Ji 1999; Alessio et al. 2000; Apor and Radi 2006; Finaud et al. 2006; Bailey et al. 2011). There is compelling evidence that this accumulation of RONS brought about by exercise is physiologically important because it activates post-exercise gene expression (Xie et al. 1999; Li et al. 2003; Murphy et al. 2004; Silveira et al. 2006; Murphy et al. 2008; Powers et al. 2010) and promotes muscle fatigue (Reid et al. 1992; Andrade et al. 1998; Reid 1998; Reid 2001; Allen et al. 2008; Bruton et al. 2008; Ferreira and Reid 2008). This is supported in part by the observation that antioxidants attenuate muscle gene expression post-exercise (Murphy et al. 2008; Petersen et al. 2012) and delay muscle fatigue in vitro and in vivo (Supinski et al. 1997; McKenna et al. 2006) in both rodent (Shindoh et al. 1990) and human muscle (Reid et al. 1994; Travaline et al. 1997; Medved et al. 2004; McKenna et al. 2006), whereas oxidants such as hydrogen peroxide increase fatigue (Andrade et al. 1998; Reid 2008).

On the basis of studies performed in vitro, RONS have been shown to have the potential to affect the function of proteins involved in many aspects of muscular contraction including the ryanodine-sensitive calcium release channel (Abramson and Salama 1989), calcium ATPase (Moopanar and Allen 2005), several structural and contractile proteins such as myosin, actin and troponin (Mishima et al. 2005; Moopanar and Allen 2006; Ferreira et al. 2008; Mollica et al. 2012), and many enzymes including glycogen phosphorylase (Cappel and Gilbert 1986). Although the mechanisms whereby RONS affect muscle function are still poorly understood, there is evidence that the reversible oxidation/nitrosylation of the
thiol residues of the amino acid cysteine in muscle proteins can affect protein function (Powers and Jackson 2008), thus enabling RONS to act as a signaling mechanism affecting a wide range of cellular functions (Ferreira et al. 2008).

Until recently, the evidence that the thiol oxidation of the above mentioned muscle proteins increases during contraction was largely based on experiments performed with purified preparations of these proteins (Allen et al. 2008) or in situ using electrically stimulated muscle preparations (McArdle et al. 1999). Recently, however, with the help of a new technique that allows the direct quantification of both reduced and oxidised protein thiols of muscle extracts (Chapter two) (Armstrong et al. 2011), we reported for the first time that a non-fatiguing bout of moderate intensity exercise, as well as high intensity exercise to fatigue in rats results in a muscle fibre-specific change in the level of thiol oxidation of several contractile proteins both during and after exercise. In particular, we showed that high intensity swimming to fatigue increases the level of protein thiol oxidation in the active red gastrocnemius muscles. This raises the question of whether a similar response is seen in humans. Since this issue has never been addressed before, the primary purpose of this study was to examine the effect of a maximal sprint on the level of protein thiol oxidation of muscle proteins in humans. Based on the previous work on rats, it is hypothesised that a maximal sprint exercise to fatigue will result in an increase in the level of thiol oxidation of muscle proteins that returns progressively back toward pre-exercise levels during recovery.
4.2 MATERIALS AND METHODS

4.2.1 Reagents

Unless otherwise stated, all chemicals and reagents were obtained from Sigma Aldrich (Castle Hill, Australia). Ethanol (99.5%) and acetone (99.5%) were purchased from Redox chemicals. Double deionised water was used throughout. Protein molecular weight standards were purchased from BioRad, Australia. BODIPY FL-N-(2-aminoethyl) maleimide (FLm) and texas red maleimide (TRm) were purchase from Invitrogen, Australia. Muscle biopsy needles and all medical supplies were obtained from The Australian Medical Association.

4.2.2 Participants

Five healthy males (see table 4.1) were recruited from the student population of the University of Western Australia (UWA). Participants were informed of all the risks associated with the study and gave written informed consent. Ethical approval was obtained from the UWA Human Research Ethics Committee. Prior to testing, participants were required to fast overnight and to minimise their physical activity and not to ingest any vitamin supplements, caffeine, alcohol or drugs over the 24-hour period preceding testing.

4.2.3 Exercise testing

Participants were required to attend the UWA Human Performance Laboratory on three separate occasions. The first visit was a familiarisation session during which anthropometric measurements were taken (height, weight, sum of six skin folds). Then, participants were subjected to an incremental exercise test to determine their maximal oxygen consumption
(VO₂ max), and after adequate rest were required to complete a 30-second all out maximal sprint on a Repco front access cycle ergometer.

The second visit to the laboratory took place at least seven days after the familiarisation session. During this session, participants were asked to attend the laboratory after an overnight fast. Resting blood samples and a muscle biopsy was taken after the participant had rested for at least 15 minutes. Then, five minutes after a light three-minute warm up at an intensity of about 100 Watts (W), each participant was asked to stand on the pedals with their dominant leg in front while supporting their weight on the handlebars and pedals. After a countdown, each participant was required to perform a 30-second all out maximal sprint, while strong verbal encouragement was provided throughout. Immediately post-exercise, a muscle biopsy was taken from the vastus lateralis muscle. Muscle biopsies were taken again 15 and 40 minutes later while the participant rested. Blood samples were taken at 5, 15, 25 and 40 minutes into the recovery period. After performing the final muscle biopsy and blood sample, the participant was required to perform another 30-second all out sprint as described above.

On the third visit to laboratory, the subject completed the exercise test exactly as described above, with the exception, that no blood or muscle biopsy samples were take. In order to estimate indirectly muscle oxygenation levels, the upper leg of each participant was fitted with a near infrared spectroscopy probe, and NIRS measurements of oxyhemoglobin, deoxyhemoglobin, and tissue oxygen saturation were recorded throughout the entire exercise test and recovery period. These measurements were taken on a separate visit to the laboratory since they could not be taken at the same time as the muscle biopsy procedure.
4.2.4 Muscle biopsy sampling

Muscle biopsies were performed using a microbiopsy needle as described by others (Hayot et al.). Muscle samples were taken from the middle third of the vastus lateralis muscle, just anterior to the iliotibial band. Two muscle samples were taken from each thigh, with each biopsy site separated from the other by at least 10 cm as recommended by Costill and colleagues (Costill et al. 1988). After injection of 2% lidiocaine, a small incision was made and the biopsy needle was inserted. Approximately 10-15 mg of muscle tissue was removed and immediately freeze clamped and wrapped in pre-cooled aluminum foil before being kept in liquid nitrogen until storage in a -80°C freezer.

4.2.5 Blood sampling

Prior to each blood sample, one of the participant’s hands was placed in a bag and pre-heated in warm water (47°C) for five minutes in order to arterialise venous blood. Blood was sampled from the participants finger and immediately analysed using a blood gas analyser (Radiometer, Copenhagen, Denmark) for the measurement of pH, pO2, pCO2, SO2, total hemoglobin concentration (ctHb), glucose and lactate levels (Table 4.2).

4.2.6 Near infra-red spectroscopy

Near infra-red spectroscopy (NIRS) has been used extensively to measure indirectly muscle oxygenation level in vivo (Hamaoka et al. 2011). NIRS measurements were taken using a portable NIRS device (Portamon, Artenis Medical System, Netherlands) and associated software (OxySoft version 2.1.6). The Portamon was paired with a computer using bluetooth and data was acquired in real time at a sampling rate of 10 Hz. Before placement
of the device, the skin was thoroughly shaved to decrease interfering noise during measurement. The device was placed on the *vastus lateralis* muscle belly between the muscle biopsy sites to ensure that the data from the Portamon were from the same part of the active muscle as that biopsied. A black elastic thigh support collar was used to hold the Portamon device securely in place and to prevent any outside light from interfering with the data. Oxyhemoglobin, deoxyhemoglobin, total hemoglobin and tissue saturation index were recorded for five minutes prior to exercise as well as throughout exercise and 40 minutes of recovery. Changes in oxyhaemoglobin, deoxyhaemoglobin and total haemoglobin levels were measured using the differences in absorption characteristics of light at 750 and 860 nm. Muscle oxygen saturation index or percentage of oxyhaemoglobin in oxygenated form was automatically calculated using the provided software (Fujimoto and others 2007; Hamaoka *et al.* 2007).

4.2.7 Dual labelling technique for the quantification of reduced and oxidised protein thiols

**Sample Preparation**

Approx 5-10 mg of muscle sample was crushed with a pestle and mortar submerged in liquid nitrogen before being placed and weighed in a centrifuge tube pre-cooled in liquid nitrogen. Then, ice cold 20% TCA/acetone (w/v) was added in the ratio of 1 ml/10 mg wet weight muscle powder. The sample was immediately homogenised (Ultra-Turrax T25 - Rose Scientific) at maximal intensity for 30 seconds to produce an homogenous suspension, then incubated for at least one hour at -20°C to precipitate proteins. In order to remove the TCA from the extract, a 250 μl aliquot was taken and mixed with 1 ml of pure
acetone (pre-cooled to -20°C). The sample was vortexed and centrifuged for five minutes at 8000 g at 4 °C. The resulting supernatant was removed, leaving the protein pellet undisturbed.

Labelling of reduced thiols

After TCA removal, the resulting protein pellet was solubilised in 50 μl of SDS buffer (0.5% SDS, 0.5 M Tris, pH 7.5), and 6 μl of 5 mM FLm was immediately added. In order to completely solubilise this protein pellet, it was subjected to 5 x 5-second periods of sonication, then vortexed for 20 minutes. Care was taken during sonication to avoid frothing. The samples were centrifuged, and the supernatant removed. After the first solubilisation, a small yellow pellet remained, so another 50 μl of SDS buffer was added together with 4 μl of 5 mM FLm, the sample was again sonicated and vortexed as before until the entire pellet was solubilised into a clear solution. Then, the supernatants from both solubilisation procedures were combined into the original tube to ensure maximal protein recovery, and the FLm labelling reaction was allowed to continue for 30 minutes at room temperature in the dark, as the fluorescent tags are light sensitive. After this and in order to remove the un-reacted FLm, 1 ml of ethanol pre-cooled to -20°C was added to the FLm-labeled protein extract, briefly vortexed, and incubated at -20°C for at least one hour to precipitate proteins. Samples were then centrifuged for five minutes (8000g, 4°C), and the supernatant containing the un-reacted dye was removed, leaving the resulting protein pellet undisturbed. The ethanol rinse was repeated once more, and the protein pellet was re-suspended in 60 μl of SDS buffer and vortexed until completely dissolved.
At this stage, all samples were standardised based on their FLm (i.e. reduced protein thiol) concentration in order for samples to be read at similar points along the fluorescent standard curves, thereby improving precision. Furthermore, since muscle samples exist in a predominant reduced state (approximately 90% reduced), the FLm concentration provides an accurate approximation of protein concentration, thereby allowing the standardization of the sample to a similar protein concentration for SDS PAGE. Samples were assayed for FLm by aliquoting 10 µl of sample into a 1.5 ml centrifuge tube and diluting it with 310 µl of 0.1 M NaOH. An aliquot (100 µl in triplicate) was read against an FLm standard curve using a fluorescent plate reader (Fluostar Optima: BMG Labtech – Germany), with the excitation wavelength set at 485 nm and emission wavelength set at 520 nm. All samples were diluted to the same FLm concentration (approximately 50 µM) using SDS buffer.

A 50 µl aliquot of the FLm-labeled sample was reduced with the addition of 4 µl of SDS buffer containing 50 mM TCEP (pH 7.0). The sample was briefly vortexed, then incubated for 60 minutes at room temperature in the dark. After reduction, TRm labelling was performed by first diluting the TCEP concentration of the sample with the addition of 50 µl of SDS buffer (to avoid TCEP interference with TRm labelling). Then, 5 µl of 5 mM TRm was added, the sample was vortexed briefly, then incubated for 60 minutes at room temperature in the dark. To improve protein band separation during SDS PAGE, a 20 µl aliquot was removed prior to TRm removal, and subjected to SDS PAGE (see below).

To the remaining sample, excess TRm was then removed with the addition of 400 µl of ethanol and this mixture was incubated for at least 60 minutes at -20°C to precipitate proteins. Samples were then centrifuged for five minutes (8000g at 4°C) and the
supernatant and excess dye were discarded. The sample was re-suspended in 100 µl of SDS buffer and the ethanol rinsing procedure was performed twice more. After the third rinse, the remaining protein pellet was re-suspended in 100 µl of SDS buffer before being assayed for FLm and TRm.

A standard curve for FLm and TRm was constructed by adding 16 µl of 1.5 mM dye to 384 µl ovalbumin solution (20 mg/ml in SDS buffer) and then diluting with SDS buffer. The assay was performed by diluting 10 µl of sample or standard with 310 µl of 0.1 M NaOH, and 100 µl was assayed. Fluorescence was measured using a fluorescent plate reader (Fluostar Optima; Flm: ex 485 nm, em 520 nm; TRm: ex 595 nm, em 610 nm). The concentrations of FLm and TRm in the samples were calculated from a second order polynomial standard curve. The percentage of oxidised protein thiols was calculated from the equation: TRm concentration/(TRm concentration + Flm concentration) x 100.

4.2.8 Protein assay

The final protein concentration of all samples was determined using a modified BioRad DC protein assay. The working reagent A’ and the sample were diluted two fold with double deionised water. The assay was performed by pipetting 20 µl of sample, 70 µl of reagent A’ and 170 µl of reagent B into a clear flat bottom 96-well plate. Absorbance was measured at 750 nm and the protein concentration of samples was calculated using linear regression.
4.2.9 SDS PAGE

Sample preparation for gel electrophoresis

To quantify the reduced and oxidised thiols of specific protein bands, and to determine muscle fiber type composition, one dimensional SDS PAGE was used to separate protein bands according to their molecular weight. In order to perform this, a 20 μl aliquot of the dual labeled protein sample was taken prior to TRm removal and combined with 4 μl of 2 mM cysteine to quench any unreacted TRm. Then, 12 μl of gel sample buffer (125 mM Tris, p.H 6.8, 4% SDS, 30% (v/v) glycerol, 0.02% bromophernol Blue, 0.4 M dithiothreitol) was added. 30 μl of sample (approximately 7.5 μg protein) was added to the gel.

Gel and buffer compositions and electrophoresis protocol

Gel electrophoresis was performed using the BioRad Mini Protean III system. Gel and buffer compositions were based on those described previously [24], with the difference that a 12% polyacrylamide gel was used and 5 mM DTT was added to the top running buffer. Electrophoresis was carried out at 8 mAmmps with voltage not exceeding 150V for 20 hours at 4 °C.

Gel analysis

The gel was scanned (Typhoon Trio, GE Health, Australia) for fluorescence (FLm: ex 485 nm, em 520 nm; TRm: ex 595 nm, em 610 nm). The bands were quantified by densitometry using image J version 1.41 software (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA) using the integrated density function. To assess the level of protein thiol oxidation of specific protein bands, dominant bands were
enclosed with a box, then the signal from the FLm and TRm channel was taken from exactly the same portion of the protein band by alternating between channels. A percentage measure was calculated by using the equation \((\text{TRm signal}/(\text{TRm} + \text{FLm signal}) \times 100)\). In order to account for inter-gel variations between FLm and TRm fluorescent scans, a gel standard was run on each gel. To ensure standards and samples were of a similar relative intensity after fluorescent scanning, FLm and TRm standards from the 96 well plate assay described above, were mixed in a ratio of one part TRm standard to four parts FLm standard. An adjustment factor for each gel was calculated based on the relative intensity of the FLm and TRm signals seen from the scans. After the gels were scanned, the molecular weight of major bands was calculated by matching band positions with protein molecular weight standards (BioRad, kaleidoscopoe pre-stained molecular weight marker), which were clearly visible at the TRm wavelength.

To assess muscle fiber-type composition, SDS PAGE was used. The myosin heavy chain band, with a molecular weight of approximately 220 kDa (Kohn and Myburgh 2006) consistently divided into three distinct bands. A plot profile was taken of the three myosin isoform bands, and the peak optical density was used to calculate relative fibre-type composition as described previously by others (Talmadge and Roy 1993; Taylor et al. 1997; Bamman et al. 1999; Kohn and Myburgh 2006) (Fig. 4.9). It is important to note that in order to clearly separate the myosin heavy chain protein band into three distinct isoforms, electrophoresis was carried out for 20 hours. This resulted in a loss of low molecular weight proteins such as a troponin, which were not examined in this study.
4.2.10 Statistical analysis

All data is presented as means ± SEM. Means were compared using a t-test or one-way ANOVA with repeated measures where appropriate, with post hoc analysis performed using a Dunnet test. Significance was established a priori at p < 0.05.

4.3 RESULTS

4.3.1 Descriptive characteristics of the participants

Table 4.1: Participant descriptive characteristics (n = 5).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>24.4 ± 1.6</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>180.6 ± 2.6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.7 ± 2.1</td>
</tr>
<tr>
<td>Sum of 6 skinfolds</td>
<td>53.6 ± 8.0</td>
</tr>
<tr>
<td>Cycle VO$_2$max (ml·kg$^{-1}$·min$^{-1}$)</td>
<td>53.7 ± 3.9</td>
</tr>
<tr>
<td>Type I fibres (%)</td>
<td>40.5 ± 15.7</td>
</tr>
<tr>
<td>Type II fibres (%)</td>
<td>59.5 ± 15.7</td>
</tr>
</tbody>
</table>

4.3.2 The effect of exercise on power output and blood variables

In response to an all out cycling effort, maximum power (1057 ± 56 W) was achieved within five seconds, then steadily declined to approximately 60% of peak power after 30 seconds (Fig. 4.1), indicating marked muscle fatigue by the end of the sprint. Consistent
with the high intensity exercise, blood pH fell and lactate concentration increased markedly (Table 4.2).

Table 4.2. The effect of a 30-second sprint on blood gas variables.

<table>
<thead>
<tr>
<th>Recovery (min)</th>
<th>pH</th>
<th>pCO₂ (mmHg)</th>
<th>pO₂ (mmHg)</th>
<th>cHb (g/dL)</th>
<th>SO₂ (%)</th>
<th>Glu (mM)</th>
<th>La (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest 0.01</td>
<td>7.42 ± 0.01</td>
<td>40.1 ± 1.31</td>
<td>91.58 ± 8.10</td>
<td>17.42 ± 0.57</td>
<td>97.36 ± 0.33</td>
<td>5.38 ± 0.19</td>
<td>0.64 ± 0.09</td>
</tr>
<tr>
<td>5 0.02</td>
<td>*7.24 ± 0.02</td>
<td>*32.3 ± 1.69</td>
<td>111.55 ± 13.34</td>
<td>16.62 ± 0.81</td>
<td>95.96 ± 1.13</td>
<td>5.36 ± 1.14</td>
<td>*7.9 ± 1.70</td>
</tr>
<tr>
<td>15 0.01</td>
<td>*7.31 ± 0.01</td>
<td>*33.56 ± 2.19</td>
<td>76.86 ± 4.38</td>
<td>16.34 ± 0.29</td>
<td>94.7 ± 0.40</td>
<td>*6.74 ± 0.26</td>
<td>*5.6 ± 0.24</td>
</tr>
<tr>
<td>25 0.01</td>
<td>*7.36 ± 0.01</td>
<td>*34.48 ± 1.33</td>
<td>89.88 ± 11.36</td>
<td>16.22 ± 0.52</td>
<td>94.96 ± 0.51</td>
<td>6.28 ± 0.56</td>
<td>*4.3 ± 0.70</td>
</tr>
<tr>
<td>40 0.01</td>
<td>7.41 ± 0.01</td>
<td>*35.4 ± 2.36</td>
<td>73.4 ± 3.45</td>
<td>15.92 ± 0.30</td>
<td>95.54 ± 0.55</td>
<td>6.06 ± 0.50</td>
<td>*2.84 ± 0.46</td>
</tr>
</tbody>
</table>

* Indicates significantly different from rest value. (p < 0.05). Results expressed mean ± S.E. (n = 5).

4.3.3 The effect of exercise on the level of thiol oxidation of muscle proteins

In response to exercise, the level of protein thiol oxidation in total protein declined from 9.5 ± 0.7% at rest to 6.9 ± 0.9% (p < 0.01) immediately after exercise (Fig 4.2.1). No significant difference was detected during that time for total thiols (p = 0.58, Fig 4.2.2) and free thiols (p = 0.28, Fig 4.2.3). Oxidised thiols decreased from 3.4 ± 0.26 nmol/mg at rest to 2.57 ± 0.32 nmol/mg (p < 0.01) immediately after exercise (Fig. 4.2.4).
The level of thiol oxidation of three proteins (myosin, actin, and glycogen phosphorylase) were quantified using 1D SDS PAGE. Following the maximal sprint, there was a 39% decrease in myosin oxidation (Fig. 4.3.2) and a 7.3% decrease in glycogen phosphorylase oxidation state (Fig. 4.3.3) relative to resting values. There was no significant change in the thiol oxidation of actin (Fig. 4.3.4). Altogether, the specific protein and total protein thiol oxidation measurements indicate that the decrease in the level of protein thiol oxidation following exercise was likely a consequence of the conversion of oxidised to reduced protein thiols.

In order to determine if the fall in the level of protein thiol oxidation in response to exercise is related to a decrease in muscle oxygenation, NIRS was used to estimate the change in oxygen tissue saturation. In response to high intensity exercise, the tissue saturation index decreased from 62.2 ± 2.3% at rest to 33.2 ± 1.8% by the end of the 30-second maximal sprint (Fig. 4.4). No differences were seen in power data between the sprints performed for the muscle biopsy and NIRS procedures, indicating a similar physiological effort of subjects between tests.

4.3.4 The effect of post-exercise recovery on power output and blood variables

After 40 minutes of recovery, blood pH had returned to pre-exercise levels, but both blood lactate and pCO2 were still above resting values (Table 4.2). After 40 minutes of recovery, muscle contractile capacity was fully recovered since there was no significant difference between the first and second maximal sprint effort relative to peak power (Fig. 4.5.1), total
work (Fig. 4.5.2), and power expressed as a percentage of maximal peak power at any time point (Fig. 4.5.3).

### 4.3.5 The effect of recovery from exercise on the level of thiol oxidation of muscle proteins

During the post-exercise recovery period, the level of protein thiol oxidation increased from 6.9 ± 0.8 % immediately after exercise to 12.1 ± 1.6 % and 12.1 ± 1.1 % after 15 and 40 minutes of recovery respectively (Fig. 4.6.1). At 15 and 40 minutes into recovery, the level of protein thiol oxidation was significantly higher than before exercise (9.5 ± 0.7 %). There was no significant difference in total protein thiols (Fig. 4.6.2) or reduced protein thiols (Fig. 4.6.3) at any point during recovery. Oxidised protein thiols were significantly higher at 40 minutes into recovery but not at 15 minutes (Fig. 4.6.4).

The level of thiol oxidation of myosin (Fig. 4.7.2) and glycogen phosphorylase (Fig. 4.7.3) were both significantly elevated at 15 minutes post-exercise compared to those prior to and immediately after exercise. In contrast, the level of thiol oxidation of actin was not significantly different from resting levels at any point during recovery. Altogether, the specific and total protein thiol oxidation results indicate that the increase in total protein thiol oxidation post-exercise was likely a consequence of the conversion from reduced to oxidised protein thiols.

### 4.3.6 The effect of recovery from exercise on muscle oxygenation level

In order to determine if muscle oxygen concentration is related to the level protein thiol oxidation post-exercise, oxygen content in muscle was estimated indirectly using NIRS.
During recovery from exercise, tissue saturation index increased and peaked within one minute after exercise, then slowly declined to levels at 15 minutes that remained stable for the remainder of the recovery period (Fig. 4.8).
Fig 4.1. The effect of a 30-second maximal cycling effort on power output (Fig. 4.1.1) and power relative to peak power (Fig. 4.1.2). Results expressed as mean ± SEM (n = 5).
**Fig. 4.2:** The effect of a 30-second maximal cycling effort on the percentage of protein thiol oxidation (Fig. 4.2.1), total protein thiols (Fig. 4.2.2), free thiols (Fig. 4.2.3) and oxidised thiols (Fig. 4.2.4). Results are expressed as mean ± SEM (n = 5). Nmol/mg refers to soluble protein. * Indicates significantly different from rest value (p < 0.05).
Fig. 4.3: The effect of a 30-second maximal cycling effort on the percentage of thiol oxidation of the whole gel lane (Fig.4.3.1), myosin (Fig.4.3.2), glycogen phosphorylase (Fig. 4.3.3), and actin (Fig. 4.3.4). Results expressed relative to resting level (%). Results are expressed as mean ± SEM (n = 5). * Indicates significantly different from rest value (p < 0.05).
**Fig. 4.4:** The effect of a 30-second maximal cycling effort on tissue saturation index (TSI) at rest and at the completion of the sprint. Results expressed as mean ± SEM (n = 5). * Indicates significantly different from rest value (p < 0.05).
Fig 4.5: The effect of 40 minutes of recovery on the peak power (Fig. 4.5.1), total work (Fig. 4.5.2), and power relative to peak power (Fig. 4.5.3) associated with the first and second 30-second maximal sprint effort. Results expressed as mean ± SEM (n = 5). No significant difference observed between the first and second sprint.
Fig. 4.6: The effect of 40 minutes of recovery from a 30-second maximal sprint effort on the percentage of muscle protein thiol oxidation (Fig. 4.6.1), total protein thiols (Fig. 4.6.2), free thiols (Fig. 4.6.3), and oxidised thiols (Fig. 4.6.4). Results are expressed as mean ± SEM (n = 5). Nmol/mg refers to soluble protein. * Indicates significantly different from 0 value.
Fig. 4.7: The effect of 40 minutes of recovery from a 30-second maximal sprint effort on the percentage of thiol oxidation of the whole lane (Fig. 4.7.1), myosin (Fig. 4.7.2), glycogen phosphorylase (Fig. 4.7.3), and actin (Fig. 4.7.4). Results are displayed relative to pre-exercise resting levels. Results expressed as mean ± SEM (n = 5). * Indicates significantly different from sprint value (0 recovery) (p < 0.05).
Fig. 4.8: Fig. 4.8.1: The effect of 40 minutes of recovery from a 30-second maximal sprint effort on tissue saturation index (TSI). Fig. 4.8.2: Representative trace of tissue saturation index (green), oxyhemoglobin (red) and deoxyhemoglobin (blue). Periods 0-1, 1-2, 2-3 and 3-4 correspond to resting, warm-up, 30-second maximal sprint, and recovery, respectively. Results expressed as mean ± SEM (n = 5). * indicates significantly different from immediate post-exercise value (p < 0.05).
**Fig. 4.9:** Representative image of FLm (Fig. 4.9.1) and TRm (Fig. 4.9.2) gel scans. Representative image of myosin heavy chain separation (Fig. 4.9.3) and plot profile (Fig. 4.9.4).
4.4 DISCUSSION

The effect of exercise on the level of thiol oxidation of muscle proteins has never been examined before in humans. This is the first study to address this issue and to show that the level of thiol oxidation of muscle proteins decreases in response to a maximal sprint effort. This study also shows that muscle protein thiol oxidation increases above resting levels during the post-exercise recovery period. As discussed later, these findings have the potential to be important for our understanding of the involvement of muscle protein thiol oxidation in many aspects of muscle function related to exercise, including optimal force production (Reid 2001), fatigue (Ferreira and Reid 2008) and contraction-induced adaptive responses to exercise-training (McArdle et al. 1999; Powers et al. 2011).

The finding that the level of thiol oxidation of muscle proteins, including that of myosin and glycogen phosphorylase decreases in response to a maximal sprint effort may appear to be inconsistent with the multiple studies which have shown that exercise increases oxidative/nitrosative stress (Davies et al. 1982; Jackson et al. 1985; Halliwell and Gutteridge 1989; Sumida et al. 1989; Jackson 1994; Sen et al. 1994; Sen 1995; Van Der Meulen et al. 1997; Ashton et al. 1998; Ashton et al. 1999; Bejma and Ji 1999; Alessio et al. 2000; McArdle et al. 2001; Gomez-Cabrera et al. 2003; Medved et al. 2004; Vasilaki et al. 2006; Ferreira and Reid 2008). It is important to note, however, that most studies have investigated exercise of much longer duration and of much lower intensity than in the current study (Sumida et al. 1989; Ashton et al. 1998; Ashton et al. 1999; Bejma and Ji 1999; Alessio et al. 2000; McArdle et al. 2001; Gomez-Cabrera et al. 2003; Medved et al. 2004), with oxidative stress ascertained based mainly on blood markers rather than on muscle analyses (Sumida et al. 1989; Ashton et al. 1998; Ashton et al. 1999; Alessio et al.
2000). Also, since muscle sampling has in general not been performed immediately after exercise in many studies (Sen et al. 1994; Van Der Meulen et al. 1997; McArdle et al. 2001; Vasilaki et al. 2006), they may have failed to detect any transient decline in intramuscular markers of oxidative/nitrosative stress. Here, in contrast, our participants were subjected to a short-duration maximal sprint effort, with muscle samples taken before and immediately post-exercise. Also, and more importantly, comparing these findings with those of others is limited by the fact that none of the other studies examined the effect of exercise on the level of protein thiol oxidation of muscle proteins in humans.

It is important to stress that although this is the first study to show that a maximal sprint effort results in a transient decline in the level of thiol oxidation of muscle proteins in humans, a similar pattern of response in the soleus muscle of rats recovering from a short bout of high intensity exercise was found in chapter three. However, it is noteworthy that not all skeletal muscles in rats respond in a similar way to high intensity exercise given the evidence that this response is muscle fibre dependent. Indeed, we have shown that this type of exercise in rats has no effect on the level of thiol oxidation of proteins in the type IIb fibre-rich white gastrocnemius muscle and results in a transient rise in protein thiol oxidation level in the red gastrocnemius, a muscle rich in type IIa fibers (Chapter three). These findings in rats therefore raise the issue of whether this could also be the case in humans, with protein thiol oxidation levels falling during intense exercise in type I muscle fibres while remaining stable or increasing in type II muscle fibers. Since the muscle samples obtained from of our participants were comprised of a mixture of type I and II fibers (Table 1), it is possible that the fall in protein thiol oxidation level in our muscle samples represents the net balance between muscle fibre specific fall and rise in thiol
oxidation levels. Until the protein thiol oxidation level of individual muscle fibres is examined in humans, this possibility remains unresolved.

The mechanisms underlying the decline in the level of muscle protein thiol oxidation during maximal exercise remains to be elucidated. One possibility is that the fall in protein thiol oxidation reflects a rapid and transient decrease in muscle RONS generation during intense contraction. However, since there is evidence that hypoxia stimulates, rather than decreases, RONS production in skeletal muscle at rest and during exercise (Bailey et al. 2001; Clanton 2007), interpretation must be reconciled with the fall in muscle oxygenation during high intensity exercise seen in the present study as well as reported by others (Kozlov et al. 2005). One possibility is that a hypoxia-mediated stimulation of RONS generation is compensated by a more pronounced decrease in RONS production by mitochondria during exercise due a shift in mitochondria respiratory state from state four (basal) to state three (maximal ADP-stimulated respiration). That this might be the case is suggested by the work of Chance and colleagues who showed that mitochondrial production of RONS is higher in state four, typical of resting muscle, than in state three, which is the state associated with muscular contraction (Boveris and Chance 1973; Chance et al. 1979; Papa et al. 1997; Vina et al. 2000). Irrespective of whether this mechanism holds, it does not explain the absence of any fall in the thiol oxidation level of actin, despite being present in the same intracellular compartment as myosin. It is possible that this is related to the observation that despite its five thiol groups, actin is less sensitive to redox modification because in its polymerized form F-actin (major form in skeletal muscle) is protected to a large extent against changes in thiol oxidation level (Liu et al. 1990).
Clearly, more research is required to elucidate the mechanisms underlying the sprint-mediated responses of protein thiol oxidation level reported here.

Irrespective of the mechanisms involved, the sprint-mediated decrease in protein thiol oxidation level described in this study raises the issues of the impact that this may have on muscle contractility. Based on the work of Reid and colleagues, this fall in the level of muscle protein thiol oxidation may contribute to the onset of muscle fatigue associated with sprinting. This interpretation is based on the observation, using isolated muscle preparations and muscle bundles, that thiol reducing agents cause a decrease in contractility in un-fatigued muscle, but increase force production in fatigued muscle (Reid et al. 1992; Reid et al. 1993). These observations have led to the view that RONS have a biphasic effect on contractility, and there is an optimal level of RONS for maximum contractility (Reid 2001) below or above which muscle force is impaired (chapter one Fig. 1.1). If this were to be the case here, the fall in the level of thiol oxidation of total and specific muscle proteins below optimal levels would be expected to contribute to muscle fatigue.

The marked increase in protein thiol oxidation during recovery from sprinting in humans is also another novel observation. One may argue that this post-exercise rise in protein thiol oxidation is consistent with the many studies reporting increased oxidative stress during recovery from exercise in humans (Gomez-Cabrera et al. 2003; Steinberg et al. 2006). In particular, the results of the present study are supported by previous research conducted by Groussard and co-workers (Groussard et al. 2003) who showed an increased in RONS as measured by electron spin resonance spectroscopy in blood that peaked at twenty minutes into recovery following the same exercise protocol used in the present study. However,
none of the above mentioned studies have examined the level of protein thiol oxidation during recovery from exercise. The only other study that has examined this issue was that performed on rats in chapter three where the level of protein thiol oxidation in the red *gastrocnemius* muscle was found to increase during the recovery period from high intensity exercise.

The mechanism underlying the post-exercise increase in the level of muscle protein thiol oxidation remains to be determined. It is possible that it involves changes in oxygen concentration as NIRS measurements show that oxygen saturation increased and remained elevated above resting levels for up to 40 minutes post-exercise (Tschakovsky and Sheriff 2004). This post-exercise increased oxygenation may cause an increase in RONS production and protein thiol oxidation. Another mechanism involves the conversion of xanthine dehydrogenase to xanthine oxidase by exercise (Vina et al. 2000). As a result, this increase in xanthine oxidase activity would be expected to cause the degradation of hypoxanthine to xanthine, resulting in the production of RONS (Hellsten *et al.* 1997; Vina et al. 2000; Volek et al. 2002) that in turn have the capacity to oxidise the thiol groups of muscle proteins. Evidence that xanthine oxidase is potentially an important source of RONS production post-exercise is suggested by the observation that the administration of allopurinol, a xanthine oxidase inhibitor, reduces oxidative stress during sustained exhaustive exercise in humans (Gomez-Cabrera *et al.* 2003; Gomez-Cabrera *et al.* 2005; Gomez-Cabrera *et al.* 2008).

The post-exercise transient increase in protein thiol oxidation may be of physiological significance as there is evidence that RONS production in response to exercise plays an
important role in controlling skeletal muscle adaptation to training (Davies et al. 1982; Gomez-Cabrera et al. 2008; Powers and Jackson 2008). For instance, contraction-mediated RONS production has been reported to be favourable to mitochondrial biogenesis (Boveris and Navarro 2008), as well as the up-regulation of anti-oxidant enzymes (Ji 1993; Leeuwenburgh et al. 1997; McArdle et al. 2001; Radak et al. 2008) and heat shock proteins (Khassaf et al. 2001; McArdle et al. 2001; Morton et al. 2006) to protect muscle cells against future oxidative damage. The importance of this mechanism is further supported by the observation that the use of antioxidants during and after exercise may negate some of the beneficial adaptations to training (Peterson and Guttridge 2008; Petersen et al. 2012).

In conclusion, the results of the present study show that sprinting causes a transient reduction in the level of thiol oxidation of muscle proteins in general as well as that of specific proteins such as myosin and glycogen phosphorylase. This is then followed by a rapid increase in protein thiol oxidation level above resting levels. We propose that these patterns of changes during and after exercise may play some role in muscle fatigue and muscle adaptation to training, respectively.

4.5 ACKNOWLEDGEMENTS

This research was supported by an Australian Research Council Linkage Grant. Muscle biopsies were performed by Dr Arnish Singh.
CHAPTER 5

GENERAL DISCUSSION
5.1 INTRODUCTION

This PhD research project has led to the development of a dual labelling technique for the quantification of the oxidation state of protein thiols in biological tissues. Based on the results of this thesis and others studies performed in our lab (El-Shafey et al. 2011), this technique appears to provide a more sensitive biomarker of oxidative stress than other markers commonly used such as the carbonyl and MDA assays. This is in agreement with the prediction made by others that RONS may cause reversible oxidative modification to protein thiols at lower concentrations than those required to cause irreversible oxidative damage (Jones 2006; Jones 2008). Furthermore, the dual labelling technique was better able to detect significant differences between biological populations than the commonly used Ellman’s assay of reduced thiols. This is significant, especially considering that the dual labelling technique for the quantification of the oxidation state of protein thiols can be applied to a wide range of biological tissues and physiological processes in the field of redox biology.

The dual labelling technique developed in this thesis has allowed for the first time the investigation of the role of protein thiol oxidation in muscle function in vivo. This is of significance since it has been postulated that protein thiol oxidation is involved in the control of many aspects of muscle function including optimal contractility, fatigue, recovery and adaptation of muscle to training. In particular, this thesis has explored the effect of exercise and recovery on the thiol oxidation of skeletal muscle proteins in rats and humans in vivo. As a result, exercise and recovery have been shown to cause marked
changes in protein thiol oxidation levels with complex differences in the extent and directional change of individual protein bands.

Since this PhD has two parts: (1) The development of the dual labelling technique of protein thiol measurement and (2) the application of the technique in animal and human models of exercise, the general discussion is presented in two separate sections. The first discusses issues in the measurement of protein thiols and how these were overcome during the development of the dual labelling technique. The second part discusses the application of the technique to the muscles of both rats and humans subjected to exercise and recovery.

5.2 OVERVIEW OF THE DEVELOPMENT OF THE DUAL LABELLING TECHNIQUE USED FOR THE MEASUREMENT OF FREE AND OXIDISED PROTEIN THIOLS IN SKELETAL MUSCLES

In marked contrast to the numerous studies which have focused on irreversible oxidative/nitrosative damage, the investigation of reversible protein thiol oxidation in tissue has been hampered by the lack of suitable analytical techniques (Makmura et al. 2001; Jones 2006; Reid 2008; Hawkins et al. 2009; Powers et al. 2010) due mainly to the reactivity of protein thiol groups (Jacob et al. 2003). Indeed, the oxidation of thiol groups by ambient molecular oxygen can occur rapidly and spontaneously (Creighton 1984; Hansen and Winther 2009), possibly resulting in artefactual oxidation during tissue collection, sample preparation and analysis. For these reasons the challenges posed by the development of the dual labelling technique described in this thesis included the prevention of artefactual protein thiol oxidation and protein loss during (a) the collection and storage
of tissue sample, (b) the extraction of tissue proteins, (c) the labelling of the reduced thiol groups, (e) the reduction of the oxidised thiols and (f) the subsequent labelling of newly reduced (previously oxidised) thiols.

The first challenge to address protein thiol measurement is to prevent oxidation of tissue samples during sample collection. To the best of my knowledge, no study has described how rapidly changes in protein thiol oxidation occur after tissue sampling. However, because of the hypoxic stress experienced by a tissue following its removal from the body, and the highly reactive nature of protein thiols, this has the capacity to affect RONS production and protein thiol oxidation. I immediately freeze clamped tissue samples with tongs pre-cooled in liquid nitrogen to minimise the possibility of oxidation occurring post sampling.

The next challenge was to both extract and denature the proteins from tissue samples so as to expose the protein thiol groups to the labelling reagents without any change in their level of thiol oxidation. Given that artefactual oxidation has been shown to be minimised under acidic conditions and low temperatures (Hansen and Winther 2009; Hill et al. 2009), proteins were extracted by mechanically disrupting each muscle sample (homogenising) in the presence of a 20% TCA-acetone solution at -20°C. The efficacy of this TCA/acetone at preventing artefactual oxidation was shown by the observation that there was less than a 3% increase in the level of thiol oxidation of samples kept for 24 hours at room temperature in the TCA/acetone solution, as opposed to an almost five fold increase in the percentage of oxidised protein thiols in samples incubated in SDS buffer pH 7.0 (Fig. 2.4.1). In this respect, I found that a combination of 20% TCA in acetone followed by incubation at -20°C
was effective to (a) precipitate and denature proteins, (b) remove potential non-protein thiol containing compounds such as GSH and GSSG, and (c) minimise oxidative side reactions.

The next challenge with the development of the dual labelling technique was to re-solubilise the proteins from the muscle extract while minimising both protein loss and artifactual oxidation during the labelling of the reduced thiol groups. This was challenging because the solubilisation of muscle proteins is difficult to achieve (Babu et al. 2004; Thierry Rabilloud et al. 2006; Bodzon-Kulakowska et al. 2007) and because incomplete solubilisation and recovery of proteins after labelling can lead to a loss of accuracy if solubilised proteins are not representative of tissue and a loss of precision if the degree of solubilisation of individual proteins varies from sample to sample. Moreover, conditions that optimise protein solubilisation can also promote protein thiol oxidation or interfere with other reagents. In this regard, I found that labelling without TCA resulted in an almost 50% increase in the estimated level of protein thiol oxidation (section 2.4.2), demonstrating that sample preparation procedures can result in artefactual oxidation in the absence of TCA pre-treatment.

In order to minimise the possibility of artefactual oxidation during protein solubilisation, protein samples were subjected to saturation labelling with maleimide (FLm) during the solubilisation process to alkylate and label the reduced thiols. The choice of a maleimide derivative as the fluorescent thiol labelling reagent was based on the fact that maleimides react rapidly and irreversibly with thiol groups and are relatively stable (Baldwin and Kiick 2011). Using this label to react with the free protein thiols, we showed effective saturation labelling and minimal non-specific interaction with proteins when the pH of the reaction is
held below 7.5 (section 2.4.1). Also, since detergents such as SDS enhance protein solubilisation and extraction and help expose the protein thiols, SDS was included in the solubilisation buffer. I found a 97% solubilisation of tissue proteins in the presence of a 0.5% SDS, 0.5M Tris buffer combined with repeated sonication, vortexing and centrifuge during saturation labelling (2.4.1). I also showed that un-reacted reagent can be effectively removed without excessive loss of protein by precipitating proteins with ethanol and removing unreacted dye in the supernatant after centrifugation.

After labelling the reduced thiols, the next challenge was to reduce the oxidised thiols and then to label them so as to provide a ratio-metric measure of the level of thiol oxidation of proteins. In order to reduce the oxidised thiols, I used TCEP because it is an effective water soluble reducing agent. The thiols, once reduced by TCEP, were then labeled with another fluorescent maleimide thiol labelling reagent (TRm) with emission/excitation characteristics different from the first (FLm) label. This dual labelling approach thus allowed the measurement of both free and oxidised protein thiols on the same tissue sample and provides a ratio-metric measure which can further improve detection limits and sensitivity. It is important to stress that with ratio-metric techniques, care must be taken to ensure that one label (FLm) does not interfere with the emission or excitation of the other label (TRm), so the correct selection of fluorescent probes is essential. I tested the interference between the wavelengths of the two fluorescent maleimide probes used in our assay and no significant interference was detected within normal working range (up to 30 uM concentration). As far as I am aware, this dual labelling technique is the first one to directly measure free and oxidised protein thiols on the same complex muscle sample that is suitable for in vivo application and can be extened using SDS PAGE.
Another important advantage with the dual labelling technique described above is that the labeled proteins can be subjected to one dimensional gel electrophoresis to quantify the level of thiol oxidation of individual protein bands, with proteins identified with mass spectrometry. This approach is particularly useful for identifying protein targets affected by reversible thiol modification and allows the quantification of the oxidation state of a large number of protein bands simultaneously. In this regard, I was able to identify myosin, actin, troponin and glycogen phosphorylase using 1D SDS PAGE.

In conclusion, part one of this thesis describes the development of a novel fluorescent dual labelling technique to quantitatively measure both reduced and oxidised protein thiols on the same tissue sample. This ratio-metric measure is independent of protein concentration and is sensitive enough to detect small changes in the level of protein thiol oxidation. Finally, in combination with gel separation, the measurement of both reduced and oxidised thiols can be performed at the level of individual proteins.

5.3 EFFECT OF EXERCISE AND RECOVERY ON THE LEVEL OF PROTEIN THIOL OXIDATION OF MUSCLES IN RATS

The newly developed two tag technique for protein thiol measurement provided the opportunity to investigate the effect of exercise and recovery on protein thiol oxidation level in skeletal muscle, as well as assess the effectiveness of this technique as a biomarker of oxidative stress.
5.3.1 The use of the level of protein thiol oxidation as a marker of oxidative stress

One popular approach to assess oxidative stress is to measure changes in protein carbonyl levels produced by irreversible oxidative protein damage (Berlett and Stadtman 1997; Dalle-Donne et al. 2003; Dalle-Donne et al. 2006; Levine and Stadtman 2006; Uzun et al. 2007). However, in agreement with (Jones 2008), my results suggest that protein thiol oxidation can occur at RONS concentrations lower than that required to cause irreversible protein damage, therefore providing a more sensitive biomarker of oxidative stress. This is supported by the findings in Chapter three, which demonstrated that there was no significant difference between control and exercises muscle for the protein carbonyl assay, whereas significant differences were detected using the dual labelling technique (Fig. 3.1).

Furthermore, compared to the Ellman’s assay (Ellman 1959), which has been used extensively as a biomarker of oxidative stress (Thornalley and Vasak 1985; Bindoli et al. 1988; Rahman et al. 1996; McArdle et al. 1999; Himmelfarb et al. 2000; McArdle et al. 2001; Balcerczyk et al. 2003; Allanore et al. 2004; Uzun et al. 2007) the results of this research project showed that the dual labelling technique was better able to detect significant differences in oxidative stress between biological populations as demonstrated between control and dystrophic muscles (Fig. 2.6). Furthermore, our laboratory recently reported (El-Shafey et al. 2011) that the GSH/GSSG ratio, which has also been used extensively as an indicator of oxidative stress (Kadiiska et al. 2000; Rabinovich et al. 2001; Schafer and Buettner 2001; Qi et al. 2011) and as an indirect measure of the level of protein thiol oxidation (Schafer and Buettner 2001), may not provide an accurate representation of the level of protein thiol oxidation as measured by the dual labelling technique (Ji et al. 1993; Sen 1995; Sen 1998; El-Shafey et al. 2011). This may be because GSH can be
released to the extra-cellular space (Sen 1998) and imported from plasma by exercising skeletal muscles (Ji et al. 1993).

Overall, my results indicate that the dual labelling technique may provide a more sensitive marker of oxidative stress than others such as the carbonyl or Ellman’s assays. Furthermore, where protein thiol oxidation is of interest, the GSH:GSSG ratio may not be a substitute for the direct quantification of protein thiols as measured by the dual labelling technique.

5.3.2 The effect of exercise on the level of muscle protein thiol oxidation

As discussed in chapters one, three and four, exercise results in a marked increase in the rate of RONS production in skeletal muscles (Davies et al. 1982; Finaud et al. 2006; Reid 2008). There is evidence that the resulting increase in oxidative stress plays important physiological roles such as that of mediating muscle fatigue and increasing muscle gene expression post-exercise. The mechanisms whereby exercise-mediated oxidative stress affects muscle function have been the subject of much research, with several studies performed on isolated muscle proteins suggesting that the reversible thiol oxidation of key muscle proteins may play some role (Hertelendi et al. 2008; Pinto et al. 2011; Mollica et al. 2012). What has remained unclear, is whether the level of thiol oxidation of these and other muscle proteins are affected by exercise and recovery in vivo. This lack of information has been attributed, in part, to the absence of a method sensitive enough to detect small differences in the level of thiol oxidation of muscle proteins.
Using the dual labelling technique, the effect of both a fatiguing and non-fatiguing bout of exercise on the level of thiol oxidation of muscle proteins in rats was measured to determine whether this process is affected by the pattern of muscle activity and muscle fibre composition (chapter three). The results showed that the effect of exercise on the level of thiol oxidation of total muscle proteins is muscle fibre specific. Indeed, there was a significant increase in the percentage of thiol oxidation of proteins as well as that of myosin and glycogen phosphorylase in the red gastrocnemius muscle of rats subjected to high intensity swimming, while the other proteins examined here remained unaffected. To the best of our knowledge, this is the first study to directly test and confirm the prediction that exercise causes an increase in the level of protein thiol oxidation in skeletal muscles in vivo (Reid 1998; Ferreira and Reid 2008; Ferreira et al. 2008). However, both exercise protocols had no effect on the level of protein thiol oxidation in the white gastrocnemius and resulted in a fall in the soleus muscle, possibly indicating that protein thiol oxidation in response to exercise is fibre type specific.

One disadvantage with the in vivo animal model used in chapter three to investigate the effect of exercise on the level of thiol oxidation of muscle proteins is that fatigue is difficult to quantify. In order to overcome this limitation, the effect of a 30-second maximal sprint effort on the level of thiol oxidation of muscle proteins was examined in humans (chapter four). The advantage with performing this type of study in humans is that fatigue can be measured objectively as a steady decline in performance on a cycle ergometer.

The study conducted on humans led to the unexpected discovery that in response to a 30-second maximal sprint effort, the level of thiol oxidation of total muscle proteins as well as
that of myosin and glycogen phosphorylase fell significantly (p < 0.05). These changes in the level of protein thiol oxidation were accompanied by a significant 40% fall in muscle power together with a 47% decrease in muscle oxygenation level. As discussed in chapter 4, the mechanism explaining the fall in the level of protein thiol oxidation during intense exercise remains to be elucidated.

5.3.3 Effect of recovery from exercise on muscle protein thiol oxidation level

During recovery, the level of protein thiol oxidation was expected to return to pre-exercise levels, but unexpectedly, increased above resting levels in both the rat and human models described in this thesis. These findings are of interest because of the possible involvement of muscle protein thiol oxidation as a factor affecting optimal force production (Reid 2001), fatigue (Ferreira and Reid 2008) and gene expression (McArdle et al. 1999; Powers et al. 2011).

The increase in the level of protein thiol oxidation during recovery from high intensity exercise also raises the issue of the mechanism underlying RONS production post-exercise. One possibility relates to the post-exercise hyperaemia which may have increased muscle oxygen content as suggested by the NIRS results in chapter four which showed increased muscle oxygen saturation throughout the recovery period. Since, as discussed above, the RONS generation by mitochondria and NADPH oxidase is sensitive to oxygen concentration (Munns et al. 2005; Sun et al. 2011), it is reasonable to expect that the post-exercise increase in oxygen concentration may have caused an increase in both RONS production and the level of protein thiol oxidation. Alternatively, it is also possible that the rate of RONS production by the mitochondria increased as mitochondria moved from state
III to state IV during recovery (Di Meo and Venditti 2001; Jackson 2007). Finally, the ischemic reperfusion response associated with high intensity muscle contraction may have triggered the conversion of xanthine dehydrogenase to xanthine oxidase (XOD), causing the degradation of hypoxanthine to xanthine and the production of RONS (Barnes 1980; Sjogaard et al. 1986; Hellsten et al. 1996; Vina et al. 2000; Volek et al. 2002; Slattery et al. 2012). Such a role for XOD is supported by the observation that markers of oxidative stress in response to exercise are reduced when allopurinol (a XOD inhibitor) is administered (Vina et al. 2000; Gomez-Cabrera et al. 2003). Clearly, more work is required to elucidate the mechanisms underlying the post-exercise rise in protein thiol oxidation seen in this research project.

5.3.4 Physiological significance of the effect of exercise and recovery on the level of muscle protein thiol oxidation

To my knowledge this is the first study to report that muscle protein thiol oxidation decreases in response to a fatiguing bout of maximal exercise in humans and the soleus muscle of rats. These findings raise the issue of the effect that such changes may have on muscle contractility and susceptibility to fatigue. As discussed in chapter four, I propose, based on the work of Reid and colleagues (1992, 1993), that this fall in the level of protein thiol oxidation may contribute to the onset of muscle fatigue associated with sprinting. This interpretation is based on the observation, using isolated muscle preparations and muscle bundles, that thiol reducing agents cause a decrease in contractility in non-fatigued muscle, but increase force production in fatigued muscle (Reid et al. 1992; Reid et al. 1993), thus suggesting that there is an optimal level of RONS for maximum contractility (Reid 2001) below or above which muscle force is impaired. If this were to be the case here, the fall in
the level of thiol oxidation of total and specific muscle proteins in response to high intensity exercise would be expected to decrease muscle contractility and contribute to muscle fatigue. How important such a mechanism of fatigue would be relative to those already described in the literature is a question that remains to be answered.

Some of my findings in humans and rats bring into question the importance of an increase in protein thiol oxidation as a mediator of muscle fatigue. For instance, the observation in humans that 40 minutes after sprinting, the performance of a second 30 second sprint was unimpaired compared to the first sprint, despite the level of protein thiol oxidation being elevated, brings into question the notion that increased oxidative stress is a mediator muscle fatigue. Moreover, the fact that both high intensity exercise to fatigue and moderate intensity non-fatiguing exercise in rats resulted in a similar increase in total protein oxidation state in the red gastrocnemius muscle is inconsistent with the theory that increased protein thiol oxidation is a mediator of muscle fatigue. Finally, with respect to the white gastrocnemius muscle, the absence of any increase in the level of thiol oxidation of total as well as specific proteins does not support the notion that the increased thiol oxidation of proteins contributes to muscle fatigue.

Since oxidative stress has been shown to increase gene expression (Gomez-Cabrera et al. 2005; Murphy et al. 2008; Ristow et al. 2009; Petersen et al. 2012), it is possible that the post-exercise rise in the level of protein thiol oxidation may act to stimulate gene expression, thus contributing to the adaptive changes that take place after exercise. In support of this view, contraction-mediated RONS production has been reported to be favourable to mitochondrial biogenesis (Boveris and Navarro 2008) as well as the up-
regulation of anti-oxidant enzymes (Ji 1993; Leeuwenburgh et al. 1997; McArdle et al. 2001; Radak et al. 2008) and heat shock proteins (Khassaf et al. 2001; McArdle et al. 2001; Morton et al. 2006). Exercise can also up-regulate the redox-sensitive transcription factor NF-κB (Powers et al. 2010; Petersen et al. 2012), which has been implicated for some of the adaptations of skeletal muscle to exercise (Kabe et al. 2005; Kramer and Goodyear 2007).

5.3.5 Protein thiol-specific responses to changes in oxidative stress

The findings in chapter three and four reveal that the extent and the direction of the change in the level of thiol oxidation of muscle proteins is specific to the protein being examined. It seems that for a given tissue, muscle proteins do not respond in a uniform manner to exercise and recovery, and that changes in the level of protein thiol oxidation of specific proteins in response to exercise is a complex and highly specific phenomenon. For example, in rats, the level of thiol oxidations of myosin and glycogen phosphorylase increased in response to high intensity exercise, while the oxidation states of actin and troponin remained unchanged (Fig. 3.2). In humans, exercise resulted in a decrease in the oxidation state of myosin and glycogen phosphorylase, while that of actin remained unaffected (Fig. 4.7). Furthermore, in the soleus muscle high intensity exercise resulted in a decrease in protein thiol oxidation of troponin, while moderate exercise caused an increase in protein thiol oxidation of troponin (Fig. 3.6). The differences between the thiol oxidation responses of individual proteins remain to be explained, but may reflect differences in RONS production within the cell, thiol exposure and reactivity and possible interaction with anti-oxidant systems.
5.4 LIMITATIONS

Although the dual labelling technique developed here has provided us with the capacity to measure small changes in the thiol oxidation level of total and specific muscle proteins, it is important to note that this technique suffers from some limitations. First, the combination of this technique with 1D SDS PAGE has enabled us to identify only a limited number of proteins which are not significantly contaminated by other proteins. This is an issue that could be addressed, at least in part, by using 2D gel analyses. For example, the ryanodine-sensitive Ca\textsuperscript{2+} release channel has been shown to contain thiol groups that are sensitive to oxidative modification and may well be implicated in protein thiol oxidation in response to exercise and muscle fatigue, but we were unable to identify this protein band from the major protein bands isolated using our protocol of one dimensional SDS PAGE. Other one or two dimensional approaches may be useful to identify changes in this and other proteins not examined in this project. However, proteins that exist in low concentrations such as signaling proteins including proteins kinases, phosphoprotein phosphatases and transcription factors may not be readily visible using either 1D SDS PAGE or 2D gel analyses.

In addition, a number of questions remain without an answer. In particular, the type of reversible thiol modification responsible for the oxidation of protein thiols remains to be established as the dual labelling technique does not differentiate between intra/inter-protein disulphide bond formation, nitrosylation, glutathionylation, and cysteinylation of protein thiols. Finally, since the cysteine residues on a given protein may differ in their susceptibility to oxidation, the issue of the specific thiol group modified within a particular protein and the impact that this has on protein function remains to be established.
Another limitation of the study conducted on humans relates to the relatively small sample size (n=5). Whilst I was able to demonstrate significant changes for some key variables with a sample size of five, the strength of the argument may be strengthened with a greater sample size.

**5.5 CONCLUSION**

In conclusion, this thesis has resulted in the development of a novel sensitive technique for the quantification of the oxidation state of protein thiols in biological tissues which has potential for a wide range of biological, biomedical, and clinical applications. Furthermore, I have demonstrated that exercise causes a change in the oxidation state of muscle protein thiols in both rats and humans *in vivo*, with these changes being both muscle fibre type and protein specific. I also demonstrated that following high intensity exercise, the level of protein thiol oxidation increases into the post-exercise recovery period. One important remaining challenge is to establish the roles that changes in the level of protein thiol oxidation may have on muscle contractility, fatigue, and gene expression.
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