PROTEIN THIOL OXIDATION AND MUSCLE CONTRACTILE PERFORMANCE

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EXECUTIVE SUMMARY

There is mounting evidence that reactive oxygen species (ROS) play an important role in the development of muscle fatigue. Some of the proposed mechanisms involve the reversible thiol oxidation of muscle proteins as well as ROS-induced protein carbonylation. Although the functions of several muscle proteins have been reported to be affected by these processes, it still remains to be determined whether the thiol oxidation levels of these and other proteins increase with fatiguing muscle stimulation, and whether this is an important mechanism of ROS-mediated muscle fatigue. It was the primary objective of this thesis to address these issues using isolated extensor digitorum longus (EDL) muscles from male Wistar rats, ARC(s) mice and Ins2Akita diabetic mice.

Using EDL muscle preparations from rats, this thesis provides evidence that reversible protein thiol oxidation plays a more important role than protein carbonylation in muscle fatigue. Firstly, total protein thiol oxidation level increases in response to fatiguing stimulation, but not following sustained non-fatiguing stimulation. Secondly, unlike protein carbonylation responses, the recovery of muscle contractile performance after fatiguing stimulation is accompanied by a return of total protein thiol oxidation to pre-stimulation level. Thirdly, both muscle fatigue and total protein thiol oxidation level are more pronounced when fatiguing stimulation takes place in the presence of the thiol oxidising agent, diamide. Fourthly, muscle contraction performed with the thiol reducing agent, dithiotreitol (DTT), decreases the magnitude of both muscle fatigue and total protein thiol oxidation level without affecting protein carbonylation level. The muscle
proteins targeted by reversible protein thiol oxidation include the contractile proteins, actin, myosin, troponin and tropomyosin.

One limitation with the aforementioned results is that they are based on experiments performed at 25 °C. Since this temperature is markedly different from that of working muscles, this raises the question of the importance of the thiol oxidation level of muscle proteins contributing to fatigue at physiological muscle temperature. Using isolated mouse EDL preparations incubated at 34 °C, this thesis shows that the thiol oxidation level of muscle proteins increases in response to fatiguing stimulation, and that acute exposure to DTT improves muscle contractile performance by as much as 87 %. Although this suggests that reversible protein thiol oxidation has the potential to be a major contributor of muscle fatigue at physiological temperature, the importance of this mechanism could not be ascertained because total protein thiol oxidation level in resting muscles is higher compared to in vivo muscles, suggesting DTT is beneficial, in part, because it normalises muscle function rather than only opposing muscle fatigue.

One of the issues raised by these findings is the question of whether some of the medical conditions associated with impaired muscle function also involve an increase in the thiol oxidation level of muscle proteins. This was tested using isolated muscle preparations from Ins2Akita mice. Despite the absence of difference in maximal specific force between the muscles of non-diabetic mice and diabetic mice, the latter were less resistant to fatigue. Interestingly, the finding that the total thiol oxidation level of muscle proteins attained after fatiguing stimulation in muscles from non-diabetic mice is comparable to
that of muscles from diabetic mice prior to contraction could be taken as evidence that changes in protein thiol oxidation level may not play an important role in explaining the increased fatigue response in diabetic mice. However, since the thiol oxidation level of myosin is lower in pre-fatigue muscles from diabetic mice compared to fatigued muscles from non-diabetic mice, this could explain why resistance to muscle fatigue is lower in muscles from diabetic compared to non-diabetic mice despite comparable total protein thiol oxidation level. Arguably, this interpretation holds as long as myosin responsiveness to thiol oxidation plays a more important role in determining contractile force compared with other contractile proteins such as actin, troponin and tropomyosin. Although further work is required to support this interpretation, the findings with myosin highlight the importance of examining the response of individual proteins in addition to that of total proteins.

In conclusion, this thesis shows for the first time that the thiol oxidation level of muscle proteins increases in response to fatiguing muscle stimulation, and identifies experimental conditions where oxidative stress contributes to muscle fatigue through protein thiol oxidation rather than oxidative protein damage. This thesis also suggests that reversible protein thiol oxidation has the potential to be a major mechanism of ROS-mediated muscle fatigue at physiological temperature. Finally, this thesis suggests that the increased thiol oxidation level of some proteins may explain, at least in part, the impaired muscle contractile performance associated with diseases such as diabetes.
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I would like to acknowledge and thank the following people, whose contributions have been invaluable, enriching and defining in making my PhD possible.

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<td>Description</td>
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<tr>
<td>(SR) Ca^{2+}</td>
<td>Sarcoplasmic reticulum ATPase</td>
<td></td>
</tr>
<tr>
<td>·OH</td>
<td>Hydroxyl radical</td>
<td></td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
<td></td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenyl</td>
<td></td>
</tr>
<tr>
<td>DNPH</td>
<td>Dinitrophenylhydrazine</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
<td></td>
</tr>
<tr>
<td>ecSOD</td>
<td>Extracellular superoxide dismutase</td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>Electronic spin resonance</td>
<td></td>
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<tr>
<td>FCS</td>
<td>Flavonoids from corn silk</td>
<td></td>
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<tr>
<td>FLm</td>
<td>FL-N-(2-aminoethyl) maleimide</td>
<td></td>
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<tr>
<td>FHA</td>
<td>FeSO₄, H₂O₂ and ascorbate</td>
<td></td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
<td></td>
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<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin levels</td>
<td></td>
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<tr>
<td>Hb^A1C</td>
<td>Glycated hemoglobin</td>
<td></td>
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<tr>
<td>KRS</td>
<td>Kreb’s mammalian ringer solution</td>
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<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
<td></td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate oxidase</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
<td></td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide anion</td>
<td></td>
</tr>
<tr>
<td>PLA$_2$</td>
<td>Phospholipase A$_2$</td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
<td></td>
</tr>
<tr>
<td>-SH</td>
<td>Thiol moiety</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
<td></td>
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<tr>
<td>TCEP</td>
<td>Phosphine hydrochloride</td>
<td></td>
</tr>
<tr>
<td>TRm</td>
<td>Texas red-C2-maleimide</td>
<td></td>
</tr>
<tr>
<td>T-tubule</td>
<td>Transverse tubule</td>
<td></td>
</tr>
<tr>
<td>UCP3</td>
<td>Mitochondrial uncoupling protein 3</td>
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CHAPTER 1:

LITERATURE REVIEW
1.1 INTRODUCTION

Over half a century ago, the emerging technology of electron spin resonance (ESR) spectroscopy provided the first data showing that skeletal muscle contains free radicals (Commoner et al., 1954). The biological importance of this finding was unclear at the time. It was not until the early 1980s that researchers identified the first link between muscle function and free radical biology. Electron spin resonance was again used to show that free radical content was elevated in isolated frog limb muscles stimulated to contract repetitively (Koren et al., 1980). Shortly afterwards, Davies and colleagues (1982) reported a 2- to 3-fold increase in the free radical content of skeletal muscles from rats run to exhaustion. These discoveries stimulated widespread interest in the relationship between exercise and oxidative stress.

A free radical is defined as any molecular species capable of independent existence while containing one or more unpaired electrons (Aruoma, 1994). An unpaired electron is one that occupies an atomic or molecular orbital by itself. Free radicals can be formed by the loss of a single electron or by the gain of a single electron by a non-radical molecule. The most important free radicals in the body are part of a family of molecules better known as reactive oxygen species (ROS; Cheeseman & Slater, 1993). Reactive oxygen species are chemically reactive molecules containing oxygen which include superoxide anion (O$_2^-$), hydroxyl radical (-OH) and hydrogen peroxide (H$_2$O$_2$).
1.2 DAMAGING EFFECT OF ROS AND THE ANTIOXIDANT DEFENSE SYSTEM

Reactive oxygen species have the capacity to damage proteins, cause DNA-strand breakage and compromise the integrity of polyunsaturated membrane lipids, which in turn can affect the homeostatic environment of the cell (Niess & Simon, 2007). Fortunately, all cells are endowed with a system of antioxidant enzymes that degrades ROS. The sarcoplasm contains CuZn-superoxide dismutase and glutathione peroxidase and catalase (Lawler & Power, 1998). The mitochondrial matrix contains Mn-superoxide dismutase and glutathione peroxidase. Superoxide dismutase is responsible for dismutating $\text{O}_2^-$ to form $\text{H}_2\text{O}_2$ and oxygen, while glutathione peroxidase is capable of reducing $\text{H}_2\text{O}_2$ or organic hydroperoxides to water and alcohol respectively (Lawler & Power, 1998). Catalase is able to catalyze the breakdown of $\text{H}_2\text{O}_2$ to form water and oxygen.

Other thiol-based antioxidant enzyme systems, thioredoxin and thioredoxin reductase (Nakamura, 2005) and the peroxiredoxins (Rhee et al., 2005), are also expressed in skeletal muscle (Hamelin et al., 2007). Functionally, the thioredoxin system operates as a major ubiquitous disulfide reductase responsible for maintaining proteins in their reduced state (Nakamura, 2005), while peroxiredoxins removes $\text{H}_2\text{O}_2$ and reduces both hydroperoxides and peroxynitrate with the use of electrons provided by a physiological thiol similar to the thioredoxin system (Rhee et al., 2005). Table 1.1 provides a brief overview of the important physiologic enzymatic antioxidants function of these molecules.
### Table 1.1: Important physiologic enzymatic antioxidants

<table>
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<tr>
<th>Enzymatic Antioxidants</th>
<th>Properties</th>
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<tbody>
<tr>
<td>- Superoxide dismutase</td>
<td>Located in both mitochondria and cytosol; dismutates O₂⁻ (Lawler &amp; Power, 1998)</td>
</tr>
<tr>
<td>- Glutathione peroxidase</td>
<td>Located in both mitochondria and cytosol; removes H₂O₂ and organic hydroperoxides (Lawler &amp; Power, 1998)</td>
</tr>
<tr>
<td>- Catalase</td>
<td>Located in both mitochondria and cytosol; removes H₂O₂ (Lawler &amp; Power, 1998)</td>
</tr>
<tr>
<td>- Thioredoxin and thioredoxin reductase</td>
<td>Located in both mitochondria and cytosol; thioredoxin catalyse the reduction of protein disulfide bonds, and thioredoxin active-site cysteines are regenerated by thioredoxin reductase (Nakamura, 2005)</td>
</tr>
<tr>
<td>- Peroxiredoxins</td>
<td>Located in both mitochondria and cytosol; removes H₂O₂ and organic hydroperoxides (Rhee et al., 2005)</td>
</tr>
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</table>

The function of antioxidant enzymes is complimented by lipid-soluble and water-soluble non-enzymatic antioxidants. Vitamin E, carotenes, and ubiquinol are lipid soluble and localised in cell membranes (Janero, 1991; Powers et al., 2004). Lipoate and glutathione are water soluble and widely distributed within the cytosol (Packer et al., 1995; Powers et al., 2004). Glutathione is the most abundant non-protein thiol, present at near millimolar concentrations, and is a primary determinant of the reducing environment within cells.
(Ferreira & Reid, 2008). In the context of muscle fatigue, glutathione is among the most important non-enzymatic antioxidants. These antioxidants protect the cell against ROS toxicity in several ways. These include conversion of ROS into less-active molecules (i.e., scavenging) and prevention of the transformation of less reactive ROS into more damaging forms (i.e., prevention of hydrogen peroxide transforming to the damaging hydroxyl radical). Table 1.2 provides a brief overview of the important physiologic non-enzymatic antioxidants function of these molecules.

<table>
<thead>
<tr>
<th>Enzymatic Antioxidants</th>
<th>Properties</th>
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<tbody>
<tr>
<td>• Vitamin E</td>
<td>• Lipid soluble phenolic compound; major chain breaking antioxidant found in cell membranes (Janero, 1991)</td>
</tr>
<tr>
<td>• Glutathione</td>
<td>• Nonprotein thiol in cells; serves multiple roles in the cellular antioxidant defense (Powers et al., 2004)</td>
</tr>
<tr>
<td>• alpha-Lipoic acid</td>
<td>• Endogenous thiol; effective as an antioxidant and in recycling vitamin C; may also be an effective glutathione substitute (Packer et al., 1995)</td>
</tr>
<tr>
<td>• Carotenoids</td>
<td>• Lipid soluble antioxidants located primarily in membranes of tissues (Powers et al., 2004)</td>
</tr>
<tr>
<td>• Ubiquinones</td>
<td>• Lipid soluble quinone derivatives; reduced forms are efficient antioxidants (Powers et al., 2004)</td>
</tr>
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1.3 OXIDATIVE STRESS AND BIOMARKERS

The term oxidative stress was first defined in 1985 as “a disturbance in the pro-oxidant-antioxidant balance in favor of the former” (Sies & Cadenas, 1985). Because of the complexity associated with the assessment of cellular redox balance, Jones (2006) proposed that this term should be redefined as “a disruption of redox signaling and control”. More recently, Sies and Jones (2007) defined oxidative stress as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage”.

A common approach to assess oxidative stress in biological systems involves the measurement of changes in the level of redox-sensitive molecules that respond to oxidative stress. In general, reliable markers of oxidative stress possess the following qualities, which are: 1) chemically unique and detectable, 2) increase or decrease during periods of oxidative stress, 3) possess relatively long half-lives, and 4) are not influenced by other cellular processes (Halliwell & Gutteridge, 2007).

There are many molecules that possess one or more of these attributes, and techniques are available to measure these biomarkers (Powers & Jackson, 2008). During periods of oxidative stress, pro-oxidants may overwhelm the antioxidant defenses in cells and damage cellular constituents. The oxidative stress in biological systems is characterised by the increase in the formation of radicals and other oxidants. However, due to the difficulties in measuring ROS production directly, most studies have used indirect biomarkers to demonstrate oxidative stress. These indirect biomarkers of oxidative stress...
typically fall into one of three categories 1) decrease in small-molecular-weight and/or lipid-soluble antioxidants, 2) oxidative damage to cellular components (i.e., lipids, proteins, and/or DNA), 3) disturbance in cellular redox balance (Table 1.3).

<table>
<thead>
<tr>
<th>1. Antioxidants</th>
<th>2. Oxidation products</th>
<th>3. Antioxidant/Pro-oxidant balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Glutathione</td>
<td>• Protein carbonyls</td>
<td>• Reduced glutathione to oxidised glutathione ratio</td>
</tr>
<tr>
<td>• Ascorbate</td>
<td>• Isoprostanes</td>
<td>• Cysteine redox state</td>
</tr>
<tr>
<td>• Alpha-tocopherol</td>
<td>• Nitrotyrosine</td>
<td>• Thiol/disulfide state</td>
</tr>
<tr>
<td>• Total antioxidant</td>
<td>• 8-OH-dG</td>
<td></td>
</tr>
<tr>
<td>capacity</td>
<td>• 4-hydroxy-nonenal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Malondialdehyde</td>
<td></td>
</tr>
</tbody>
</table>

There are many biomarkers to quantify oxidative stress. Unfortunately, each category of biomarkers of oxidative stress biomarkers has limitations and the development of a single and ideal biomarker has proven to be a difficult task. Hence, it appears that no one biomarker best assesses oxidative stress and that in most cases the measurement of multiple biomarkers is required to confirm the presence of oxidative stress in tissues (Halliwell & Gutteridge, 2007).
1.4  EXERCISE-INDUCED OXIDATIVE STRESS

There is compelling evidence that exercise can result in oxidative stress. These studies reveal that skeletal muscle continually generates ROS (O’Neill et al., 1996; Kolbeck et al., 1997; Stofan et al., 2000; Zuo et al., 2000; Pattwell et al., 2001; Pattwell et al., 2004). Although some studies did not show an increase in oxidative stress with exercise (Viguie et al., 1993; Sacheck et al., 2003; Ramel et al., 2004), with a decrease even reported by some (Lovlin et al., 1987; Groussard et al., 2003), a large number of studies have reported significant increases in ROS level associated with muscle contraction and exercise (Kawai et al., 1994; Laaksonen et al., 1999; Mastaloudis et al., 2001; McAnulty et al., 2003; Mastaloudis et al., 2004; Vasilaki et al., 2006; Gomez-Cabrera et al., 2010; Sahlin et al., 2010).

There may be several reasons why some investigators have failed to observe signs of exercise-induced oxidative stress. First, the use of different test subjects might have influenced the findings of different studies. There is also a lack of information on factors such as training status, age and sex which could potentially play a role in modulating oxidative stress (Vollaard et al., 2005). Furthermore, research has adopted a large range of exercise intensities. Only exercise of sufficient intensity or duration appears to lead to a large enough increase in free radical production (Poulsen et al., 1996; Quindry et al., 2003), and the sensitivity of the different markers of oxidative stress is such that some markers of oxidative stress are sensitive enough to be affected by exercise, whereas others are not (Niess et al., 1996; Liu et al., 2000).
1.5 **MECHANISMS OF ROS PRODUCTION DURING EXERCISE**

The mechanisms whereby exercise increases the production of ROS are contentious. Although various mechanisms have been proposed, their relative contributions have yet to be established. Various potential sites for ROS production exist in skeletal muscle and are summarised and discussed below (Figure 1.1).

![Diagram of ROS production](image)

**Figure 1.1. Potential sites for the production of ROS in skeletal muscle** (Powers et al., 2010). Abbreviations used: SR/T-tubule, sarcoplasmic reticulum/transverse tubule; PLA₂, phospholipase A₂; ecSOD, extracellular superoxide dismutase; MnSOD, manganese superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase.
1.5.1 Mitochondria and electron transport chain

Until recently, it was generally accepted that the most important source of ROS, both at rest and during exercise, involved an ‘electron leak’ from the electron transport chain in the mitochondrial inner-membrane (Vollaard et al., 2005). Early reports suggested that 2-5% of the total oxygen consumed by mitochondria might undergo one electron reduction with the generation of ROS (Boveris & Chance, 1973; Loschen et al., 1974). Since then, studies have identified the major site(s) of ROS generation within mitochondria, with most data now indicating that complexes I and III of the electron transport chain are the main sites of mitochondrial ROS production (Barja, 1999; Muller et al., 2004). In complex I, the main site of electron leakage to oxygen appears to be the iron-sulfur clusters, whereas in complex III, it appears to be the Q10 semiquinone (Muller et al., 2004). In complex III, the ROS is released from both sides of the inner mitochondrial membrane (Muller et al., 2004).

Until recently, it was assumed that the increased ROS generation that occurs during contractile activity is directly related to the elevated oxygen consumption which arises with increased mitochondrial activity. This implies a 50- or 100-fold increase in ROS generation by skeletal muscle during aerobic contractions (Kanter, 1994; Urso & Clarkson, 2003). St-Pierre and colleagues (2002) reassessed the rate of production of ROS by mitochondria and indicated that the upper estimate of the proportion of the electron flow that gives rise to ROS may be as low as ~0.15%. The low rate of ROS production may include a role for uncoupling proteins (specifically mitochondrial uncoupling protein 3 in
skeletal muscle) as regulators of mitochondrial production of ROS acting to protect mitochondria against oxidative damage (Brand et al., 2004; Brand & Esteves, 2005). In addition, studies reveal that mitochondria produce more ROS during state 4 (basal) respiration as compared with state 3 (maximal ADP-stimulated respiration) (Adhihetty et al., 2005; Kozlov et al., 2005). This is significant as skeletal muscle mitochondria are predominantly in state 3 during aerobic contractile activity which in turn limits their generation of ROS during contractions (Adhihetty et al., 2005; Kozlov et al., 2005; Kavazis et al., 2009). Collectively, these findings suggest that mitochondria may not be the primary source of ROS production in skeletal muscle during exercise and further studies are required to fully elucidate the role that mitochondria play in contraction-induced production of ROS in skeletal muscle (Jackson et al., 2007).

1.5.2. *Nicotinamide adenine dinucleotide phosphate oxidase*

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is another potential source of ROS production during exercise. This enzyme generates superoxide by transferring electrons from NADPH to molecular oxygen (Muller et al., 2004). Nicotinamide adenine dinucleotide phosphate oxidase exists in several cellular locations in muscle fibers (sarcoplasmic reticulum, transverse tubules, and sarcolemma) and has been postulated to contribute to exercise induced ROS production in skeletal muscle (Powers et al., 2010). The generation of ROS by NADPH oxidase has been shown at the sarcoplasmic reticulum (SR) sites of both cardiac (Cherednichenko et al., 2004) and skeletal muscle (Xia et al., 2003). The transverse tubules of skeletal muscle also contain NADPH oxidase whose
activity is increased by depolarisation (Espinosa et al., 2006; Hidalgo et al., 2006). This enzyme contains some of the classical subunits found in the NADPH oxidase of phagocytic cells, and releases superoxide to the cytosol of skeletal muscle cells (Jackson, 2008). NADPH oxidase complex is also expressed at the plasma membrane level in skeletal muscle (Javesghani et al., 2002). However, whether this complex releases ROS predominantly to the plasma membrane cannot be ascertained (Javesghani et al., 2002). Unfortunately, although NADPH oxidase is a source of ROS, the role this enzymes plays in ROS production during exercise still remains to be determined.

1.5.3. Phospholipase A$_2$-dependent processes

Phospholipase A$_2$ (PLA$_2$) is an enzyme that also has the potential to mediate ROS production during exercise. This enzyme cleaves membrane phospholipids to release arachidonic acid, which is a substrate for ROS-generating enzyme systems such as the lipoxygenases (Zuo et al., 2004). Further, activation of PLA$_2$ can activate NADPH oxidases (Zhao et al., 2002), and increased PLA$_2$ activity has been reported to stimulate ROS generation in both muscle mitochondria (Nethery et al., 2000) and cytosol, (Gong et al., 2006).

Both calcium-dependent and independent forms of PLA$_2$ are reported to play a role in muscle ROS generation (Power & Jackson, 2008). In this regard, the calcium-independent enzymes has been proposed to modulate cytosolic oxidant activity in skeletal muscle cells (Gong et al., 2006), whereas the calcium-dependent isoform located within mitochondria has been reported to stimulate mitochondrial ROS generation during contractile activity.
(Nethery et al., 1999). Gong and colleagues (2006) hypothesised that the calcium-independent PLA\textsubscript{2} is a major determinant of ROS activity under resting conditions, whereas during contractions, heat stress, or other processes elevating intracellular calcium, the calcium-dependent PLA\textsubscript{2} is activated to promote ROS production. However, the relative contribution of these enzymes to ROS production during exercise is still an unresolved issue.

1.5.4. Xanthine oxidase

Xanthine oxidase is another enzyme with the capacity to generate ROS. This enzyme oxidises hypoxanthine to produce xanthine and generate superoxide radicals (Halliwell & Gutteridge, 2007). Several studies suggest that activation of xanthine oxidase in rat skeletal muscle is an important source of contraction-induced ROS production (Judge & Dodd, 2004; Gomez-Cabrera et al., 2005; Wang et al., 2009; Gomez-Cabrera et al., 2010). However, while rat skeletal muscles contain significant levels of xanthine oxidase (Judge & Dodd, 2004), human skeletal muscle cells appear to possess low amounts of xanthine dehydrogenase or oxidase (Hellsten et al., 1996). Due to the lack of data in humans, additional research is required to determine the role that xanthine oxidase plays in exercise-induced ROS production.

1.5.5. Summary

Despite the initial indications that mitochondria are the predominant site for ROS generation during contractile activity, a number of other potential cellular sites have been
identified. It is still unclear if all of these multiple sites contribute to the increase in ROS levels during contractions or whether one site predominates. It is possible that the multiple sites of generation are active in differing situations and that the effects of the ROS generated are relatively localised and important for disparate functions.
1.6 FACTORS INFLUENCING ROS PRODUCTION DURING EXERCISE

Temperature and carbon dioxide (CO$_2$) have been identified as factors with the potential to influence ROS production during exercise.

1.6.1. Temperature and ROS production

Skeletal muscles are exposed to increased temperatures during intense exercise, particularly in high environmental temperatures. There have been many studies showing the importance of increased temperature on the production of ROS (Zuo et al., 2000; Arbogast & Reid, 2004; Moopanar & Allen 2005; Edwards et al., 2007; van der Poel et al., 2007). In general, as the temperature increases above 37 °C, the performance of isolated preparations of skeletal muscle markedly decreases, and there is strong evidence supporting the generation of ROS as a potential cause for the poor longevity of in vitro skeletal muscle at physiological temperatures (37-43 °C, Zuo et al., 2000; Edwards et al., 2007; van der Poel et al., 2002; van der Poel & Stephenson, 2002; Arbogast & Reid, 2004; Moopanar & Allen, 2005, 2006; van der Poel et al., 2007).

The effect of temperature on ROS production is likely influenced by intracellular sources. Mitochondrial oxygen consumption (Brooks et al., 1971) and the enzymatic activities of oxidoreductases, e.g., NADPH oxidase (Morgan et al., 2003) are strongly increased by a rise in temperature. Antioxidant enzyme activities are also temperature sensitive, affecting the pathways that buffer muscle-derived oxidants. Therefore, the changes in total oxidant activity likely reflect changes in the net balance of these variables.
1.6.2. **Carbon dioxide and ROS production**

During exercise, skeletal muscles are exposed to elevated CO$_2$ levels (Ranatunga et al., 1987). There is evidence suggesting that CO$_2$ influences redox homeostasis (Stofan et al., 2000; Arbogast & Reid, 2004). Carbon dioxide and bicarbonate react directly with nitric Oxide (NO) derivatives (Vesela & Wilhelm, 2002) and undergo electron exchange reactions to form carbonate radicals (Liochev & Fridovich, 2001). Dissolved CO$_2$ also promotes acidosis in biological systems, stimulating cellular production of ROS and NO (Carbonell et al., 2002). Studies in skeletal muscle about the net effect of CO$_2$-mediated reactions on ROS generation are conflicted as increased CO$_2$ is reported to both promote oxidative stress when measurement is taken outside the cell (Stofan et al., 2000) and protect against oxidative stress when measurement is taken within the cell (Arbogast & Reid, 2004).

This apparent conflict can be explained based on compartmentalisation of the two assays used in these studies (Arbogast & Reid, 2004). The reaction between CO$_2$, NO and other oxidants forms redox-active intermediates, such as peroxynitrite and carbonate radicals, that are more unstable, have shorter half-lives, and diffuse shorter distances than the parent molecules. This tends to localise nitrosative and oxidative reactions near the sites of production, which are likely to be within the cell. Thus reactions with intracellular targets become more likely, enhancing the ROS signal measured within the cell (Arbogast & Reid, 2004), and diffusion of ROS out of cells becomes less likely, lessening the signal measured outside the cell (Stofan et al., 2000).
1.6.3. Summary

In conclusion, oxidative stress during exercise is strongly influenced by temperature. Although indications that CO\textsubscript{2} could mediate in ROS production, it is still uncertain if the presence of CO\textsubscript{2} promotes or protects against oxidative stress. The limited data imply that additional research is required to determine the role of CO\textsubscript{2} in ROS production during exercise.
1.7 ROLE OF ROS IN UNFATIGUED MUSCLE

It is well established that ROS have an important influence on force production in unfatigued skeletal muscle. Low levels of ROS in skeletal muscle during basal conditions are required for normal force production (Reid, 2001; Supinski et al., 2007). This has been shown in studies using fiber bundles from rat diaphragm (Reid et al., 1993) and single fiber preparations from limb muscles of frogs (Oba et al., 1996) or mice (Andrade et al., 1998). When incubated with antioxidants such as catalase and dithiothreitol (DTT), these muscle preparations experience a decrease in early twitch force, while the initial response to \( \text{H}_2\text{O}_2 \) exposure is a rise in developed force in rat diaphragm fiber bundles (Reid et al., 1993) and in single fibers from limb muscles of frogs (Oba et al., 1996) or mice (Andrade et al., 1998). The force–frequency relationship is also depressed by ROS depletion, shifting the curve rightward in a dose-dependent manner. Furthermore, maximal tetanic force under low ROS conditions is depressed by up to 50 %, a loss that is spontaneously reversed when antioxidants are removed (Reid et al., 1993; Oba et al., 1996; Andrade et al., 1998). In contrast, exposure to a superoxide generating system stimulates a leftward shift of the force–frequency curve that is associated with an increase in twitch and submaximal tetanic forces, but with no change in maximal tetanic force (Lawler et al., 1997).

As muscular activity increases ROS levels, the redox balance is driven to an oxidised state, which also depresses force. Studies using single fibers from limb muscles of frogs (Oba et al., 1996) or mice (Andrade et al., 1998) show that prolonged exposure to an oxidant such as \( \text{H}_2\text{O}_2 \) depresses force production. Hence, the force–frequency relationship is depressed
by prolonged exposure with oxidant, shifting the curve rightward in a dose-dependent manner (Oba et al., 1996; Andrade et al., 1998). This deleterious effect of H$_2$O$_2$ is completely reversible by DTT (Oba et al., 1996; Andrade et al., 1998). It follows from these observations that there is an intermediate cellular redox state that optimises force production in skeletal muscle. This is evident when low-levels of ROS increase force in unfatigued muscle (Reid et al., 1993; Oba et al., 1996; Andrade et al., 1998).

The aforementioned findings supported the view of Reid and colleagues (1993) who proposed a theoretical model to explain the relationship between muscle redox balance and isometric force production (Figure 2). Their model predicts that the muscle redox state is a physiologically regulated variable that maintains equilibrium by matching the rates of ROS production with the cellular antioxidant capacity. This model predicts that an optimal cellular redox state exists whereby conditions are ideal for muscle force production and that a deviation from the most favourable redox balance leads to a decline in force production (Figure 1.2).
Figure 1.2. Biphasic effects of ROS in skeletal muscle force production (Reid et al., 1993). Point 1 illustrates the force generated by muscle in basal state (i.e., no antioxidants or oxidants added). Point 2 illustrates the force produced by unfatigued skeletal muscle exposed to low levels of oxidants; this represents the optimal redox state for force production. Point 3 illustrates the deleterious effects of excessive ROS on skeletal muscle force.
1.8 ROLE OF ROS AS A MEDIATOR OF MUSCLE FATIGUE

Muscle fatigue is defined as the reversible decrease in muscle force or power that occurs with muscle contraction (Allen et al., 2008). Muscle fatigue plays an important protective mechanism against ATP depletion and associated risk of rigor mortis state. Fatigue can result from many sites along the complex pathways starting in the cortex and leading to excitation of lower motor neurons in the spinal cord to the neuromuscular junction of the muscle. Fatigue resulting from processes inside the spinal cord and above is defined as central fatigue, whereas fatigue resulting from processes in the peripheral nerve, neuromuscular junction, and muscle is defined as peripheral fatigue (Allen et al., 2008).

Peripheral fatigue can result from different type of exercise intensities. Exercise that requires intense, near maximal muscle contraction results in the rapid onset of fatigue that is likely caused by metabolic stresses (Allen & Westerblad, 2001). Force declines rapidly under these conditions and recovers within seconds-to-minutes after exercise stops. In contrast, fatigue resulting from less-intense, submaximal contractions is believed to result, in part, from metabolic stresses such as exercise-mediated depletion of muscle glycogen stores (Ortenblad et al., 2011), reduced availability of blood glucose due to a fall in hepatic glycogen level (Callow et al., 1986), increased inorganic phosphate levels (Westerblad et al., 2002), hyperthermia and dehydration (Nybo, 2008) to list a few. This is the type of fatigue where oxidative stress appears to be important and will be explored in more detail.
1.8.1. Animal studies

As discussed previously, there is ample evidence that ROS production increases in working muscle and that strenuous exercise leads to oxidative stress (Kawai et al., 1994; Laaksonen et al., 1999; Mastaloudis et al., 2001; McAnulty et al., 2003; Mastaloudis et al., 2004; Vasilaki et al., 2006; Gomez-Cabrera et al., 2010; Sahlin et al., 2010). Many animal studies implicate ROS as a contributor to contraction-induced muscle fatigue (Table 1.4). Several studies using pharmacologic antioxidants have established a cause-and-effect link between increased ROS production and fatigue of exercising respiratory and limb muscles.

For instance, ROS-selective antioxidant enzymes and other antioxidant drugs have been shown to slow mechanical losses during electrically stimulated fatigue of muscle preparations in vitro (Shindoh et al., 1990; Reid et al., 1992a; Diaz et al., 1994; Khawli & Reid, 1994; Moonpanar & Allen, 2005; Ferreira et al., 2009) and in in vivo experiments (Novelli et al., 1990; Davis et al., 2009; Yu et al., 2010; Agten et al., 2011; Hu & Deng, 2011). It is important to note, however, that antioxidant efficacy depends on the fatigue protocol. Fatigue is delayed in the presence of antioxidants during contractile protocols that use submaximal activation patterns, but not when contraction is at maximal or near-maximal intensity (Reid et al., 1992; Matuszczak et al., 2005).
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Performance Indicator</th>
<th>Performance increases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shindoh et al (1990)</td>
<td>150 mg/kg N-acetylcysteine</td>
<td>Fatigue for 20 min at 20 Hz using electrically stimulated diaphragm muscle (<em>In vitro</em>)</td>
<td>21</td>
</tr>
<tr>
<td>Reid et al (1992a)</td>
<td>$5 \times 10^2$ U/ml Superoxide dismutase, $1.8 \times 10^4$ U/ml catalase</td>
<td>Fatigue for 5 min at 30 Hz using electrically stimulated diaphragm muscle (<em>In vitro</em>)</td>
<td>20</td>
</tr>
<tr>
<td>Diaz et al (1994)</td>
<td>4 mg/ml N-acetylcysteine</td>
<td>Fatigue for 4 min at 40 Hz using electrically stimulated diaphragm muscle (<em>In vitro</em>)</td>
<td>35</td>
</tr>
<tr>
<td>Khawli &amp; Reid (1994)</td>
<td>10 mM N-acetylcysetine</td>
<td>Fatigue for 10 min at 30-40 Hz using electrically stimulated diaphragm muscle (<em>In vitro</em>)</td>
<td>Significant</td>
</tr>
<tr>
<td>Moopanar &amp; Allen (2005)</td>
<td>5 mM Tiron</td>
<td>Fatigue for 2 min at 100 Hz using electrically stimulated flexor digitorum brevis (<em>In vitro</em>)</td>
<td>40</td>
</tr>
<tr>
<td>Ferreira et al (2009)</td>
<td>10 mM L-2-oxothiazolidine-4-carboxylate (OTC)</td>
<td>Fatigue for 5 min at 50 Hz using electrically stimulated diaphragm muscle (<em>In vitro</em>)</td>
<td>13-16</td>
</tr>
<tr>
<td>Novelli et al (1990)</td>
<td>6.5 mM Vitamin C and spin trappers</td>
<td>Time to exhaustion using swimming mices (<em>In vivo</em>)</td>
<td>50, 32-86</td>
</tr>
</tbody>
</table>
### Table 1.5

<table>
<thead>
<tr>
<th>Authors</th>
<th>Treatment</th>
<th>Outcome Description</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davis et al (2009)</td>
<td>12.5-25 mg/kg quercetin × 10 days</td>
<td>Maximal endurance exercise performance using running mice (<em>In vivo</em>)</td>
<td>36-37</td>
</tr>
<tr>
<td>Yu et al (2010)</td>
<td>500-2000 mg/kg flavonoids × 10 days</td>
<td>Maximal endurance exercise performance using swimming rats (<em>In vivo</em>)</td>
<td>8-62</td>
</tr>
<tr>
<td>Agten et al (2011)</td>
<td>150 mg/kg N-acetylcysteine</td>
<td>Maximal diaphragm force production using breathing mice (<em>In vivo</em>)</td>
<td>Significant</td>
</tr>
<tr>
<td>Hu &amp; Deng (2011)</td>
<td>100-400 mg/kg flavonoids daily for 28 days</td>
<td>Time to exhaustion using running mice (<em>In vivo</em>)</td>
<td>42-72</td>
</tr>
</tbody>
</table>

### 1.8.2. Human studies

Several human trials have reported that nutritional antioxidants such as vitamins C and E, beta-carotene, and linoleic acid do not improve exercise performance and are not effective ergogenic aids (Snider et al., 1992; Alessio, 1993; Rokitzki et al., 1994; Goldfarb, 1999; Avery et al., 2003; Bryant et al., 2003; Jackson et al., 2004; Powers et al., 2004; Gaeini et al., 2006). However, the antioxidant N-Acetylcysteine (NAC) has been shown to be effective in preventing muscle fatigue in humans (Table 1.5). N-Acetylcysteine, a reduced thiol donor that supports glutathione resynthesis (Ruffmann & Wendel, 1991), not only inhibits muscle fatigue of isolated muscle preparations (Shindoh et al., 1990; Diaz et al., 1994; Khawli & Reid, 1994), but also delays muscle fatigue in human experiments.
involving electrically stimulated fatigue of limb muscle (Reid et al., 1994), breathing against an inspiratory resistive load (Travaline et al., 1997), cycling exercise (Medved et al., 2004; McKenna et al., 2006; Kelly et al., 2009; Bailey et al., 2011), repeated knee extensions (Koechlin et al., 2004) and repetitive handgrip exercise (Matuszczak et al., 2005). The NAC-mediated improvements in performance range from 15 % (Reid et al., 1994; Matuszczak et al., 2005) to 69 % (Bailey et al., 2011). Nonetheless, it is noteworthy that NAC does not appear to retard muscle fatigue during exercise at near-maximal intensities in human studies where ROS effects will be minimal (Medved et al., 2003; Matuszczak et al., 2005).

Recently, it has been reported that supplementation with the flavonoid antioxidant, quercetin, may also delay muscle fatigue and improve human exercise performance (Table 1.5; Davis et al., 2010; Nieman et al., 2010). Flavonoids from corn silk (FCS) have been investigated and confirmed to possess various pharmacological activities such as antihypertensive, anti-infectious, anti-oxidative and anti-diabetic (Li & Yu, 2009; Liu et al., 2011). In recent studies, FCS has been shown to be a potent scavenger of hydroxyl and peroxyl radicals (Ren et al., 2005; Ebrahimzadeh et al., 2008; Hu et al., 2009). It has also been reported that FCS had anti-fatigue activity (Hu et al., 2010). Overall, although there is compelling evidence that antioxidant treatment can delay fatigue in healthy humans, it is clear that the efficacy depends upon the chemical properties of the antioxidant.
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Performance Indicator</th>
<th>Performance increases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reid et al (1994)</td>
<td>150 mg/kg/hr NAC for 60 min pre-exercise</td>
<td>Repetitive electrical stimulation of tibialis anterior for 30 min at 10 Hz</td>
<td>15</td>
</tr>
<tr>
<td>Travaline et al (1997)</td>
<td>150 mg/kg/hr NAC for 60 min pre-exercise</td>
<td>Time to exhaustion from breathing against inspiratory resistor</td>
<td>50</td>
</tr>
<tr>
<td>Koechlin et al (2004)</td>
<td>1800 mg/day NAC × 4 days then 600 mg NAC pre-exercise</td>
<td>Time to exhaustion from repetitive knee extensions</td>
<td>5</td>
</tr>
<tr>
<td>Medved et al (2004)</td>
<td>150 mg/kg/hr NAC for 15 min then 25 mg/kg/hr NAC pre-exercise and during exercise</td>
<td>Time to exhaustion from cycling</td>
<td>26</td>
</tr>
<tr>
<td>Matuszczak et al (2005)</td>
<td>150 mg/kg/hr NAC for 45 min pre-exercise</td>
<td>Time to exhaustion from repetitive isometric handgrip</td>
<td>15</td>
</tr>
<tr>
<td>Mckenna et al (2006)</td>
<td>125 mg/kg/hr NAC for 15 min then 25 mg/kg/hr NAC pre-exercise and during exercise</td>
<td>Time to exhaustion from cycling</td>
<td>24</td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Intervention Details</td>
<td>Outcome</td>
<td>Study Duration</td>
</tr>
<tr>
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<td>----------------</td>
</tr>
<tr>
<td>Kelly et al (2009)</td>
<td>1800 mg NAC 45 min pre-exercise Breath against inspiratory resistor for 30 min</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Davis et al (2010)</td>
<td>2 × 500 mg/day quercetin × 7 days Time to exhaustion from cycling</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Nieman et al (2010)</td>
<td>1000 mg/day quercetin × 14 days Treadmill running for 12 min</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Bailey et al (2011)</td>
<td>125 mg/kg/hr NAC for 15 min then 25 mg/kg/hr NAC pre-exercise and during exercise Time to exhaustion from cycling</td>
<td></td>
<td>24-69</td>
</tr>
</tbody>
</table>
1.9 MECHANISMS AND SITES OF ROS ACTION

The intracellular mechanism(s) by which ROS contribute to muscle fatigue are unclear. Two possible mechanisms involving the effect of ROS on these muscle proteins involved in the control and activation of muscle force production during the excitation-contraction coupling are described in the next sections. During contraction, calcium (Ca$^{2+}$) ions are released from the sarcoplasmic reticulum. These Ca$^{2+}$ ions then binds to Troponin-C, and this induces a conformational change in the regulatory complex exposing the actin molecule for binding to the myosin ATPase located on the myosin head. These changes result in the actin and myosin filaments sliding past each other thereby shortening the sarcomere length resulting in a contraction.

1.9.1. Protein thiol oxidation

One mechanism whereby ROS might play a role in muscle fatigue involves the reversible oxidation of protein thiols. The thiol moiety (-SH) of the amino acid cysteine can undergo reversible, covalent reactions with muscle-derived oxidants to form, for instance, disulfide bonds (Eaton, 2006). Thiol oxidation can alter protein function by interfering with the enzyme active sites or by altering protein structure (Ferreira & Reid, 2008). Numerous proteins involved in muscle contraction can undergo reversible thiol oxidation, and this can potentially cause muscle fatigue as summarised in the following diagram (Figure 1.3). For example, Ca$^{2+}$ handling proteins, such as the SR Ca$^{2+}$ ATPase and the SR Ca$^{2+}$ release channel have received attention because their activity can be affected by the oxidation of their thiol groups (Scherer & Deamer 1986; Abramanson & Salama, 1989; Viner et al.,
1996; Anzai et al., 2000; Pessah & Feng, 2000; Gutierrez-Martin et al., 2004; Aracena-Parks et al., 2006; Zissimopoulos et al., 2007).

Figure 1.3. Reversible thiol oxidation of muscle proteins which potentially cause muscle fatigue.

Thiol oxidation of contractile proteins could also contribute to muscle fatigue. Exposure of actin to ROS, leads to the oxidation of cysteine 374 results in structural alterations of the actin molecule (Dalle-Donne et al., 2003b), disrupting the interactions between actin and actin binding proteins (Milanzi et al., 2000). Thiol oxidation of myosin and actin decreases Ca$^{2+}$-activated force and its Ca$^{2+}$ sensitivity through impaired Ca$^{2+}$ regulation of the actin–
myosin cycle (Hertelendi et al., 2008). The oxidation of myosin causes a marked decrease in myosin ATPase activity (Root & Reisler, 1992; Prochniewicz et al., 2008a). This is a consequence of cysteine 707 oxidation in the myosin head which results in a decreased fraction of myosin heads that undergo the force-generating transition during the actomyosin ATPase cycle (Tiago et al., 2006). The effects of oxidation on actin in the actomyosin complex are predominantly determined by oxidation-induced changes in myosin heads, and changes in weak-to-strong structural transitions in actin and myosin are coupled to each other and are associated with oxidative inhibition of muscle contractile force (Prochniewicz et al., 2008a; Pizarro & Ogut, 2009).

In addition to actin and myosin, thiol oxidation of troponin (Putkey et al., 1993; Sousa et al., 2006; Pinto et al., 2008; Pinto et al., 2011) and tropomyosin (Williams & Swenson, 1982) also affects muscle contractile function. Oxidation of cysteine 98 on troponin reduces the binding affinity of troponin for actin, which destabilises the troponin-actin relationship (Pinto et al., 2011) and results in a loss of force production (Sousa et al., 2006; Pinto et al., 2008). For tropomyosin, a disulfide bridge can be formed on cysteine 190 in response to oxidation, which results in reduced binding affinity of tropomyosin to actin, which is reported to affect the ATPase activity of actomyosin (Williams & Swenson, 1982).

As mentioned above, numerous proteins involved in muscle contraction can undergo reversible thiol oxidation. However, the myofilaments may be a more physiologically important target for ROS during muscle fatigue compared to alternation of SR function, as there are indications that intracellular Ca^{2+} handling proteins are less sensitive to the
effects of increased ROS exposure compared to the contractile proteins. In these experiments, the oxidant H$_2$O$_2$ was found to decrease myofilament Ca$^{2+}$ sensitivity in single fast twitch fibers, without affecting SR Ca$^{2+}$ release, and these changes were largely reversible by exposure to the antioxidant DTT, indicating that thiol group modification was playing a major role in these effects (Andrade et al., 1998; Lamb & Posterino, 2003; Posterino et al., 2003). Endogenous ROS-induced during muscle fatigue were also shown to decrease myofilament Ca$^{2+}$ sensitivity in single fast twitch fibers, without altering SR Ca$^{2+}$ handling, and again these effects could be inhibited by DTT exposure (Moopanar & Allen, 2006).

One major limitation with all of the aforementioned studies examining the effect of ROS on muscle proteins is that these studies have been performed in vitro with isolated proteins and most have used unphysiological levels of oxidants that may not reflect physiologically relevant conditions. To date, no study has examined the effect of fatiguing contraction on the thiol oxidation levels of muscle proteins, thus leaving unanswered the identity of the protein targeted by exercise-mediated oxidative stress. This gap in our knowledge is primarily due to the difficulty of investigating changes in the thiol oxidation level of muscle proteins due to the poor sensitivity of the assay techniques available. However, a newly developed assay, referred to as the ‘2 TAG’ protein thiol method (Armstrong et al., 2011), has been shown to be sensitive enough to examine the thiol oxidation levels of muscle proteins, and, as discussed later, this method will be adopted in this thesis to identify the protein targeted by exercise-mediated oxidative stress.
1.9.2. **Protein oxidative damage**

Another possible intracellular mechanism by which ROS could contribute to muscle fatigue involves protein dysfunction caused by oxidative damage. Protein oxidative damage is generally considered to be irreversible and occurs when proteins directly react with ROS, leading to the formation of protein derivatives or peptide fragments containing carbonyl groups, such as aldehydes and ketones (Barrerio & Hussain, 2010). Increased protein oxidative damage (as measured by the carbonyl assay) has been observed during exercise and muscle contraction (Reznick et al., 1992; Saxton et al., 1994; Radák et al., 1998; Barrerio et al., 2006; Pinho et al., 2006; Matsunaga et al., 2008; Veskoukis et al., 2008; Dubersteina et al., 2009; Agten et al., 2011). An improvement in contractile force is also observed with decreased protein carbonylation following the administration of antioxidants (Reznick et al., 1992; Barrerio et al., 2006; Agten et al., 2011).

Numerous proteins involved either directly, or indirectly, in muscle contraction can undergo irreversible oxidative damage, and potentially cause muscle fatigue. For example, oxidation of the SR Ca\(^{2+}\)-ATPase can inhibit SR Ca\(^{2+}\)-pumping function, with an increase in protein carbonylation levels being associated with a decrease in Ca\(^{2+}\)-ATPase activity (Matsunaga et al., 2008). In addition, exposure of actin to ROS causes a rapid increase in the carbonylation level of actin which results in strong inhibition of actin polymerization and a complete disruption of actin-filament organization (Dalle-Donne et al., 2001; Fedorova et al., 2010). Myosin is also targeted by ROS-induced carbonylation in skeletal and cardiac muscles. The increase in the carbonylation of myosin is associated with
impaired contractile performance in cardiac muscle (Coirault et al., 2007; Shao et al., 2010) and respiratory muscle (Yamada et al., 2007). Finally, carbonylation of tropomyosin can also contribute to contractile dysfunction (Canton et al., 2006).

Although protein oxidative damage is generally considered to be irreversible (Barrerio & Hussain, 2010) and for this reason not considered to be a mediator of muscle fatigue, this view has recently been challenged by the observation in skeletal muscle and in cell cultures of pulmonary smooth muscle cells that a rise in protein carbonylation level in response to oxidative stress can be short lived (less than 30-60 min; Matsunaga et al., 2008; Wong et al., 2008, 2010), with the decarbonylation of proteins occurring independently of proteosomal activity (Wong et al., 2008), but via a mechanism which remains to be elucidated (Wong et al., 2008). These observations raise the question of whether reversible carbonylation could also be involved in promoting muscle fatigue. If protein decarbonylation post-fatiguing muscle contraction were to occur in parallel with recovery from fatigue, this could be taken as evidence that protein carbonylation may not be only a marker of muscle damage, but also a mediator of muscle fatigue. In this regard, it is noteworthy that the work of Matsunaga and colleagues (2008) shows that the recovery of Ca$^{2+}$ ATPase activity after exercise parallels that of the changes in its carbonylation rather than thiol oxidation level.

Although it is possible that a short-lived transient rise in protein carbonylation might contribute to fatigue, one major limitation with current literature is that the relative importance of this mechanism compared to reversible protein thiol oxidation has never
been examined before. Since the possible involvement of reversible protein carbonylation as a mediator of muscle fatigue still remains to be assessed, one of the objectives of this thesis is to address these issues.

1.10 STATEMENT OF PROBLEM AND HYPOTHESES

As discussed above, it still remains to be determined whether the thiol oxidation level of muscle proteins increases with fatiguing muscle contraction. For this reason, the first objective of this thesis was to establish the extent to which the rise in protein thiol oxidation levels correlates with muscle fatigue induced in response to a submaximal muscle contraction protocol.

Given the importance of controlling for muscle damage and the possibility that short-lived transient rise in protein carbonylation might also mediate fatigue, the second objective of this thesis was to examine the response of protein carbonylation to fatiguing muscle contraction.

Since temperature plays an important role in the production of ROS, the third objective of this thesis was to examine the role of protein thiol oxidation on muscle fatigue of isolated skeletal muscle preparation at 34 °C.

Finally, as a means to explore whether muscle contractile dysfunction associated with a number of medical conditions involve the reversible thiol oxidation of muscle proteins, the last objective of this thesis was to provide evidence that muscle contractile dysfunction in
Ins2Akita diabetic mice is associated with an increase in the thiol oxidation level of muscle proteins.

Using the aforementioned newly developed ‘2 TAG’ protein thiol assay method (Armstrong et al., 2011), this thesis proposes to test the following hypotheses:

**Chapter 2**

- The fatigue resulting from the isometric contraction of rat EDL muscle *in vitro* is mediated by an increase in protein thiol oxidation rather than an increase in protein carbonylation.

- The fatigue resulting from the isometric contraction of rat EDL muscle *in vitro* is associated with an increase in the thiol oxidation levels of myosin, actin, troponin and tropomyosin.

**Chapter 3**

- The fatigue resulting from the isometric contraction of rat EDL muscle at 34 °C *in vitro* is associated with a rise in protein thiol oxidation.

- The normalisation of the thiol oxidation of muscle proteins opposes the fatigue resulting from the isometric contraction of rat EDL muscle at 34 °C

**Chapter 4**

- Protein thiol oxidation is increased before and after isometric fatigue of *in vitro* EDL muscles obtained from Akita diabetic mice
• Increased protein thiol oxidation in the EDL muscles of diabetic mice is associated with a reduced muscle contractile performance.
CHAPTER 2:

PROTEIN THIOL OXIDATION AND

MUSCLE CONTRACTILE PERFORMANCE
ABSTRACT

The aim of this study was to examine whether protein thiol oxidation or protein damage (measured by protein carbonylation) resulting from oxidative stress could contribute to muscle fatigue. *Extensor digitorum longus* (EDL) muscles from male Wistar rats (*Rattus norvegicus*) incubated in Kreb’s ringer bicarbonate solution were subjected to a fatiguing stimulation protocol which consisted of 10 min submaximal tetanic contractions of 500 ms duration at 15 s intervals at a stimulation frequency of 50 Hz. These muscles were compared to either control muscles in a rested state or muscles stimulated to contract while exposed to either a protein thiol oxidising agent, diamide, or a protein thiol reducing agent, dithiothreitol (DTT). In addition, some EDL muscles were subjected twice to the fatiguing stimulation protocol mentioned above with a 60 min rest between the two fatiguing stimulation. In response to fatiguing stimulation, contractile force fell by 50.8 ± 3.1 % (p<0.05) and this was accompanied by a 34.2 ± 7.2 % (p<0.05) increase in mean total protein thiol oxidation level. Mean actin, myosin, troponin, tropomyosin thiol oxidation levels were 22.4 ± 2.7 % (p<0.05), 55.5 ± 9.2 % (p<0.05), 34.0 ± 4.5 % (p<0.05), 29.2 ± 5.7 % (p<0.05) higher, respectively. The fall in contractile force following fatiguing stimulation in muscles treated with DTT or diamide were 19.7 ± 2.7 % (p<0.05) lesser and 19.0 ± 6.5 % (p<0.05) greater, respectively, compared to untreated stimulated muscles. Mean total protein thiol oxidation levels were 24.8 ± 8.9 % (p<0.05) lower and 42.8 ± 9.6 % (p<0.05) higher in response to DTT and diamide treatments, respectively. Mean actin, myosin, troponin, tropomyosin thiol oxidation levels were 19.9 ± 2.5 % (p<0.05), 23.0 ± 2.9 % (p<0.05), 22.4 ± 3.9 % (p<0.05), 22.5 ± 3.4 % (p<0.05) lower and 42.7 ± 7.0 % (p<0.05), 25.3
± 6.3 % (p<0.05), 34.0 ± 6.5 % (p<0.05), 26.0 ± 6.9 % (p<0.05) higher in response to DTT and diamide treatments, respectively. Mean protein carbonylation was not significantly different in response to diamide and DTT treatments. In response to the 60 min rest, contractile force recovered to 92.1 ± 1.4 % (p<0.05), and this was accompanied with a full recovery in mean total protein thiol oxidation level, while mean protein carbonylation level remained elevated. The recovery in contractile force was associated with recovery in the mean thiol oxidation levels of the actin, myosin, troponin and tropomyosin. However, only actin and myosin was associated with a full recovery. In conclusion, this study corroborates for the first time the view that oxidative stress contributes to muscle fatigue through protein thiol oxidation, and that oxidative protein damage does not necessarily contribute to muscle fatigue. These findings also raise the interesting possibility that the thiol oxidation of multiple proteins directly, or indirectly, associated with contractile force, contribute to ROS-mediated muscle fatigue.

**Keywords:** Reactive oxygen species, protein thiols, dithiothreitol, muscle fatigue
2.1 INTRODUCTION

The onset of muscle fatigue, defined as a reversible loss of muscle performance, limits locomotive performance not only of individuals engaged in competitive sporting events or recreational pursuits (Allen et al., 2008), but also of older individuals and patients with chronic diseases such as heart failure (Flynn et al., 2009), chronic fatigue syndrome (Myhill et al., 2009), and chronic obstructive pulmonary disease (Baghai-Ravary et al., 2009). Among the many factors postulated to play a role in muscle fatigue, reactive oxygen species (ROS) have been identified as potentially important contributors (Ferreira et al., 2009; Kelly et al., 2009; Reardon & Allen, 2009a; Reardon & Allen, 2009b; Agten et al., 2011). This is suggested by the marked increase in ROS production that occurs during endurance exercise (Mastaloudis et al., 2001; McAnulty et al., 2003; Mastaloudis et al., 2004; Vasilaki et al., 2006; Gomez-Cabrera et al., 2010; Sahlin et al., 2010) and from the observations that pharmacologic antioxidants slow the rate of force decline in electrically stimulated muscle preparations in vitro (Shindoh et al., 1990; Reid et al., 1992a; Reid et al., 1992b; Diaz et al., 1994; Khawli & Reid, 1994; Moopanar & Allen, 2006; Ferreira et al., 2009) and improve exercise performance in in vivo experiments (Novelli et al., 1990; Davis et al., 2009; Yu et al., 2010; Agten et al., 2011; Hu & Deng, 2011). Similarly, ingestion of some antioxidants in human participants has been shown in most, but not all studies (Jackson et al., 2004; Powers et al., 2004; Matuszczak et al., 2005) to delay muscle fatigue associated with a broad range of experimental protocols (Reid et al., 1994; Traveline et al., 1997; Koechlin et al., 2004; Medved et al., 2004; Matuszczak et al., 2005; Mckenna et al., 2006; Kelly et al., 2009; Davis et al., 2010; Nieman et al; 2010; Bailey et al., 2011).
One mechanism whereby ROS might contribute to muscle fatigue involves reversible protein thiol oxidation. The thiol moiety (-SH) of the cysteine residues of proteins can undergo reversible covalent reactions with muscle-derived oxidants to form disulfide bonds (Eaton, 2006). Since muscle fatigue is also reversible, this mechanism is generally considered to explain how ROS contribute to fatigue (Ferreira & Reid, 2008). In support of this mechanism, numerous muscle proteins have been shown to undergo reversible thiol oxidation when incubated in vitro with ROS. These include, for instance, calcium handling proteins, such as the sarcoplasmic reticulum (SR) Ca\(^{2+}\) ATPase and the SR Ca\(^{2+}\) release channel (Scherer & Deamer 1986; Abramanson & Salama, 1989; Viner et al., 1996; Anzai et al., 2000; Pessah & Feng, 2000; Gutierrez-Martin et al., 2004; Aracena-Parks et al., 2006; Zissimopoulos et al., 2007). Moreover, several contractile proteins are susceptible to thiol oxidation and thus could also contribute to muscle fatigue. For example, thiol oxidation of actin could disrupt the organisation of the actin filaments, leading to reduction in the force generating ability of the sarcomere (Milzani et al., 2000). The oxidation of myosin has been shown to inhibit myosin ATPase activity (Tiago et al., 2006). The oxidation of the thiol groups of troponin also alters its function (Putkey et al., 1993). Finally, the oxidation of tropomyosin can affect the ATPase activity of actomyosin (Williams & Swenson, 1982). It is important to stress, however, that the aforementioned effects of thiol oxidation on individual muscle proteins have been based mainly on studies performed on purified proteins, and it still remains to be determined whether the thiol oxidation of individual muscle proteins increases with muscle fatigue.
During muscle contraction, ROS also have the capacity to impair contraction by causing oxidative damage to proteins through processes such as protein carbonylation. Protein carbonylation is reported to be an unavoidable physiological consequence of aerobic activity (Dalle-Donne et al., 2003a), with increased oxidative protein damage observed during exercise and repeated muscle contraction (Reznick et al., 1992; Saxton et al., 1994; Radák et al., 1998; Barrerio et al., 2006; Pinho et al., 2006; Matsunaga et al., 2008; Veskoukis et al., 2008; Dubersteina et al., 2009; Agten et al., 2011). Nevertheless, protein carbonylation associated with protein damage is not generally considered to be contributing substantially to muscle fatigue because muscle fatigue is a reversible process and protein carbonylation is considered to be irreversible (Dalle-Donne et al., 2003a). This view, however, has recently been challenged by the observation in skeletal muscles and in cell cultures of pulmonary smooth muscle cell that the rise in protein carbonylation level in response to oxidative stress can be short lived (less than 30-60 min; Matsunaga et al., 2008; Wong et al., 2008, 2010). This decarbonylation of proteins can occur independently of proteosomal activity (Wong et al., 2008), via a mechanism which remains to be elucidated (Wong et al., 2008). These observations raise the question of whether decarbonylation could also occur following muscle fatigue. In this regard, it is noteworthy that the work of Matsunaga and colleagues (2008) shows that the recovery of Ca\textsuperscript{2+} ATPase activity after exercise parallels that of the changes in its carbonylation level. Moreover, many other proteins experience an increase in their carbonylation levels in response to muscle contraction, such as actin (Dalle-Donne et al., 2001; Dalle-Donne et al., 2007; Fedorova et al., 2010), myosin (Coirault et al., 2007; Shao et al., 2010), and tropomyosin
(Canton et al., 2006). Decreased protein carbonylation levels have also been linked to improved contractile force following the administration of antioxidants (Reznick et al., 1992; Barrerio et al., 2006; Agten et al., 2011).

Since it still remains to be determined whether the thiol oxidation of muscle proteins increases with fatiguing muscle stimulation, the primary objective of this study was to provide information about whether protein thiol oxidation could contribute to ROS-mediated muscle fatigue. Also, given the possibility that a short-lived transient rise in protein carbonylation might also contribute to fatigue, the next objective was to examine the response of protein carbonylation to fatiguing muscle stimulation. Using the isolated \textit{extensor digitorum longus} (EDL) muscle preparation as the experimental model, this study provides new evidence that protein thiol oxidation contributes, in part, to the ROS-mediated muscle fatigue associated with submaximal muscle stimulation \textit{in vitro}, with little or no role played by protein carbonylation. In addition, thiol oxidation of actin, myosin, troponin and tropomyosin have been identified as potential contributors to muscle fatigue.
2.2 MATERIALS AND METHODS

2.2.1 Animals

Male Wistar rats (*Rattus norvegicus*) weighing an average of 140 g were kept at a constant room temperature ranging between 22 and 24 °C, with free access to food and water. All experiments were approved by the Animal Ethics Committee of the University of Western Australia.

2.2.2 Intact muscle experiments

Male Wistar rats were anaesthetised with an intraperitoneal injection of pentobarbital (65 mg/kg of body weight), and both EDL muscles were carefully excised. While the first muscle was being tested, the other was left intact inside the animals and removed once experimentation with the first muscle was completed (≈10 min). Using a braided surgical silk (Size 3), one end of the muscle tendon was attached to a dual mode force transducer-servomotor (1200A Intact Muscle Test System, Aurora Scientific Inc, Canada) and the tendon of the other end of the muscle was attached to an anchoring hook. The muscle preparation was bathed in Kreb’s mammalian Ringer solution (KRS) (Composition: NaCl, 121 mM; KCl, 5.4 mM; MgSO₄, 1.2 mM; NaHCO₃, 25 mM; HEPES, 5 mM; glucose, 11.5 mM; CaCl₂, 2.5 mM at pH 7.4) gassed with 5 % CO₂ and 95 % O₂ at 25 °C throughout the experiment. A temperature of 25 °C was chosen because it is within the range of temperatures that are optimal for maintaining isometric force *in vitro* (25–30 °C; Segal et al., 1985, 1986). The muscle was then stimulated with single twitches to identify the
optimum length producing the highest force possible before experimentation. Force responses were elicited using a 701B muscle stimulator (Aurora Scientific Inc, Canada). Next, maximal force responses were obtained in response to supramaximal stimulation pulses (pulse width: 0.3 ms) delivered at 150 Hz for 500 ms with a 2 min rest between each stimulation to stabilise the force responses. The muscles were then given a 10-min rest before the commencement of the stimulation. The two submaximal stimulation protocols used in the experiments on rats were based on findings from preliminary experiments. They involved exposure to stimulation pulses (80 V and 1000 mA) delivered at 50 Hz for 500 ms with either a 15 s (fatigue) or 60 s (sustained non-fatigue) interval between contractions for 10 min. For all experiments, isolated muscles were maintained for 10 min in KRS before the commencement of each experiment. In order to study the effect of contraction on protein oxidation, isolated muscles from rats were subjected to either a fatiguing stimulation or a sustained non-fatiguing stimulation, and the levels of protein oxidation were compared to resting muscles not stimulated to contract. After 10 min of stimulation or rest, all muscles were frozen using aluminium clamps pre-cooled in liquid nitrogen and stored at -80 °C. To study the effect of recovery on fatiguing stimulation and protein oxidation, isolated muscles were subjected to two fatiguing stimulation runs each separated by a 60 min rest. Muscles were frozen and stored at -80 °C either before the first fatiguing stimulation, after the first fatiguing stimulation, after the 60 min recovery, or after the second fatiguing stimulation.

In order to study the effect of protein thiol reducing and protein thiol oxidising agents on fatiguing stimulation and protein oxidation, isolated muscles from rats were maintained
for 10 min in KRS with 5 mM dithiothreitol (DTT) or 1 mM diamide before the commencement of the fatiguing stimulation. Measurements made from the fatigued muscles exposed to DTT or diamide were then compared with fatigued muscles not exposed to any DTT or diamide during the experiment. After 10 min of fatiguing stimulation, muscles were frozen and stored as described above.

2.2.3 Skinned fiber experiments

The skinned fiber experiments undertaken in this study were similar to those described previously (Han et al. 2003). Briefly, EDL muscles from male Wistar rats were removed and placed in paraffin oil. A segment of a single muscle fiber was isolated and attached to a force transducer (SI, Heidelberg) and maintained at slack length. The fibers were then chemically skinned by exposure to a low Ca\(^{2+}\) (relaxing) solution (Solution A) containing Triton X-100 (2 % v/v) to permeabilise all cellular membrane components and eliminate Ca\(^{2+}\) transport pathways. Solution A consisted of: K\(^+\) 117 mM, Na\(^+\) 36 mM, ATP 8 mM, free Mg\(^{2+}\) 1 mM, creatine phosphate 10 mM, EGTA 50 mM, N-2-Hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES) 90 mM, NaN\(_3\) 1 mM at pH 7.1. Maximal force responses were induced by exposing the muscle fibers to Solution B, which had a similar composition to Solution A, except that the [EGTA\(^2-\)] and [CaEGTA] of Solution B were 0.4 and 49.6 mM, respectively (Lamb & Stephenson, 1990). The free [Ca\(^{2+}\)] of the solutions was calculated using a \(K_{\text{app}}\) for EGTA of \(4.78 \times 10^6\) (Fink et al., 1986). In these experiments, fibers were maintained in Solution A for 2 min and then exposed to Solution B briefly to determine maximal force. Force was then returned to baseline by transfer of the fibers to Solution A.
The fibers were then exposed to a similar set of solutions, containing either 5 mM diamide or 4 mM FeSO₄, 10 mM H₂O₂ and 25 mM ascorbate (FHA) for a similar time period, to examine the effect of the oxidants on contractile force production. Finally, the fibers were exposed to another similar set of Solution A and B containing 1 mM DTT, to examine whether any effects of the oxidants on contractile force production were reversible. The effects of the oxidising and reducing agents were measured and expressed as a percentage of the corresponding initial control maximum contractile force. All experiments were performed at room temperature (23 ± 2 °C).

2.2.4 Assay of proteins thiol oxidation levels

Protein thiol oxidation levels were determined using a fluorescent two-tag labelling technique, which involved the sequential labelling of reduced and oxidised protein thiol groups using two separate fluorescent tags on the same protein sample (Armstrong et al., 2011). Briefly, muscles were homogenised on ice with 20 % (w/v) trichloroacetic acid (TCA) in acetone using an Ultra-turax (IKA, Germany) and subsequently stored at -20 °C. The protein pellets extracted from 20 % (w/v) TCA in acetone were re-suspended in 50 μl of sodium dodecyl sulphate (SDS) buffer (0.5 % (w/v) SDS and 0.5 M Tris pH 7.0) in the presence of 0.5 mM BODIPY FL-N-(2-aminoethyl) maleimide (FLm) in the dark. All samples were sonicated and incubated for 30 min at room temperature in the dark with continuous vortexing. After centrifugation, the labelled protein solution was precipitated with cold ethanol to remove excess dye (FLm), and then re-dissolved in 50 μl of SDS buffer (pH 7.0). Samples were reduced with the addition of 4 mM tris (2-carboxy-ethyl)
phosphine hydrochloride (TCEP) and incubated for 60 min at room temperature in the dark. After reduction, samples were labelled with 0.25 mM Texas red-C2-maleimide (TRm), and incubated for 30 min at room temperature in the dark. Ethanol precipitation and re-solubilisation in SDS buffer was repeated four times to remove excess un-reacted dye (TRm). Samples were re-solubilized in 100 μL of SDS buffer, and the intensity of the fluorescence was measured for both FLm and TRm (FLm: excitation = 485 nm, emission = 520 nm and TRm: excitation = 595 nm, emission = 610 nm). Ovalbumin pre-labelled with a known quantity of FLm and TRm was used as a standard to quantify labelled protein thiols. Protein concentration (mg/ml) was determined using the Bio-Rad DC Protein Assay. The percentage of protein thiol oxidation was expressed as the concentration of oxidised thiols (TRm) to total protein thiols (FLm and TRm).

2.2.5 Electrophoretic separation and assay of ‘2 Tag’-labelled protein

To determine the levels of thiol oxidation of individual protein bands in the muscle sample, polyacrylamide gel electrophoresis was performed on the fluorescently labelled samples, prepared as described above, using the BioRad Mini Protean III system (Armstrong et al., 2011). Gel and buffer compositions were based on those described previously (Kohn & Myburgh, 2006), with the exceptions that a 12 % polyacrylamide gel was used and 5 mM DTT was added to the top running buffer. Electrophoresis was carried out at 8 mA with voltage not exceeding 150 V for 8.5 hr at 4 °C, with the buffer in the lower chamber stirred with a magnetic stirrer throughout. Upon completion of the gel electrophoretic separation of proteins, the gel was immediately scanned for fluorescence
using a Typhoon Scanner (Amersham) at the wavelengths associated with both dyes (FLm: excitation = 485 nm, emission = 520 nm and TRm: excitation = 595 nm, emission = 610 nm). The individual protein bands corresponding to actin, myosin, tropomyosin and troponin were identified at molecular weight of these proteins of 42 kD, 205 kD, 33 kD and 18 kD respectively.

2.2.6 Assay of protein carbonylation levels

Protein carbonylation levels were assayed following their reaction with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenyl (DNP) hydrazone (Levine et al., 1990; Hawkins et al., 2009). In brief, muscles were homogenised on ice with 20 % (w/v) TCA in acetone using an Ultra-turax (IKA, Germany), then the muscle extracts were precipitated by centrifugation at 8000 g for 10 min at 4 °C, with the resulting protein pellets dried and re-suspended in 10 mM DNPH in 2 M HCl and incubated for 30 min at room temperature in the dark. Proteins were later washed with ethyl acetate/ethanol (1:1) and dissolved in 6 M guanidine hydrochloride, and absorbance was measured at 370 nm. Protein concentration (mg/ml) was determined using the Bio-Rad protein assay. Carbonyl levels are expressed as nmol of carbonyl per mg soluble protein.

2.2.7 Statistical analysis

Results are presented as means ± standard error of the mean. All analyses were performed using either a Student’s paired t test or a one way ANOVA followed by Fisher LSD posteriori test, with significance at P<0.05. Sample sized is denoted by n.
2.3 RESULTS

2.3.1 The effect of fatiguing and sustained stimulation on protein oxidation

Isolated EDL muscles from rats were used to investigate protein oxidation levels after muscle stimulation. Fatiguing stimulation resulted in a 51 % decline in mean contractile force (Figure 2.1A), and a 34 % increase in mean total protein thiol oxidation level (Figure 2.1B), and no significant changes in mean protein carbonylation level (Figure 2.1C). However, when compared with pre-fatigued muscles, there was an increase in protein carbonylation level (Figure 2.2D). In response to sustained non-fatiguing stimulation, mean contractile force fell by less than 5 % (Figure 2.1A), and there were no significant changes in mean total protein thiol oxidation level (Figure 2.1B) or mean protein carbonylation level (Figure 2.1C). There was no difference in total protein thiols (Figure 2.1D) indicating that the increase in protein thiol oxidation level measured likely represents increased conversion of reduced protein thiols to oxidised protein thiols. These data are consistent with protein thiol oxidation rather than oxidative protein carbonylation contributing to the loss in muscle contractile force during fatiguing stimulation.

Whether the level of protein thiol oxidation in isolated EDL muscles at 25 °C gassed with 95 % O₂ and 5 % CO₂ was comparable to that of the in vivo EDL muscles (freshly dissected) was also examined. There was no significant difference between resting mean total protein thiol oxidation level (7.5 ± 0.3 %, n=6) and in vivo mean total protein thiol oxidation level (7.6 ± 0.2 %, n=6).
Figure 2.1. The effect of fatiguing and sustained stimulation on protein oxidation. Isolated EDL muscles were stimulated at 50 Hz for 500 ms with either a 15 s (fatigue) or 60 s (sustained) interval between contractions for a period of 10 min. Following 10 min of stimulation, mean percentage of initial force (A), percentage of protein thiols oxidised (B), protein carbonyls (C) and total protein thiols (D) were measured. *Significantly different measured at p<0.05 relative to resting muscle incubated for 10 min without stimulation. n=6. Values are expressed as means ± SE.

2.3.2 The effect of recovery from fatiguing stimulation on protein oxidation

Given that protein thiol oxidation can be reversed by intracellular antioxidant pathways, and that protein carbonylation can be short-lived, it was of interest to examine whether muscle recovery from fatigue was associated with the normalisation of protein thiol oxidation and/or protein carbonylation levels. Muscles were subjected to two 10 min
fatiguing stimulation runs each separated by a 60 min rest period. After 60 min of rest, mean total protein thiol oxidation level returned to pre-fatigue resting level (Figure 2.2C), but there was no recovery in mean protein carbonylation level (Figure 2.2D). Contractile force at the beginning of the second fatiguing stimulation recovered to 92 % of the initial contractile force attained in response to the first fatiguing stimulation (Figure 2.2A). Following the second fatiguing stimulation, the decline in contractile force was comparable to the first fatiguing stimulation (Figure 2.2B). Mean total protein thiol oxidation also increased to a level comparable to that attained after the first fatiguing stimulation (Figure 2.2C), whereas there was a further progressive increase in mean protein carbonylation level (Figure 2.2D). Taken together, these data indicate that changes in protein thiol oxidation, but not protein carbonylation, correlate with changes in muscle contractile force. Overall, there was no change in total protein thiols which indicated that reversible protein thiol oxidation could be contributing to the decrease in contractile force (Figure 2.2E).

To investigate whether increases in protein carbonylation could lead to a decrease in contractile function, isolated EDL were exposed to FHA to increase protein carbonylation level. The oxidative damage reagent caused a significant increase in mean protein carbonyls from 1.85 ± 0.2 nmol.mg⁻¹ to 3.5 ± 0.3 nmol.mg⁻¹ (n=6, P<0.05). This was associated with a 24.6 ± 2.4 % (n=6, P<0.05) decline in initial contractile force. These findings suggest that excessive protein carbonylation level can affect muscle contractile force.
Figure 2.2. The effect of recovery from fatiguing stimulation on protein oxidation. Isolated EDL muscles were fatigued by two fatiguing stimulation runs which consisted of 50 Hz for 500 ms with a 15 s interval between contractions for a period of 10 min separated by a 60 min rest. Following a 60 min rest, the recovered muscles were fatigued for a second time. Mean percentage of initial force of first fatigue (A), percentage of oxidised protein thiols (C), protein carbonyls (D), total protein thiols (E) were measured before first fatigue (rest), after first fatigue, after recovery and after second fatigue. Percentage of mean
force relative to initial force of the first fatigue (△) and second fatigue (△) were measured every min for the 10 min fatiguing stimulation (B). *Significantly different measured at p<0.05. n=6. Values are expressed as means ± SE.

2.3.3 The effect of protein thiol modification on fatiguing stimulation and protein oxidation

To provide further evidence that reversible protein thiol oxidation contributed to the loss of contractile force in response to 10 min of fatiguing stimulation, an investigation was conducted to examine whether the protein thiol reducing agent, DTT could prevent the loss in muscle contractile force. DTT did not affect the mean initial absolute force (Figure 2.3A), but following 10 min of fatiguing stimulation, the mean force of muscles exposed to DTT was 20 % higher than muscles not exposed to DTT (Figure 2.3B). The improvement in contractile force was associated with a 25 % reduction in mean total protein thiol oxidation level (Figure 2.3C), but no change in mean protein carbonylation level (Figure 2.3D).

If reversible protein thiol oxidation contributed to the loss in contractile force, then the protein thiol oxidising agent diamide should augment the loss in muscle contractile force. Diamide did not affect the initial absolute force (Figure 2.3A), but following 10 min of fatiguing stimulation, the mean force of muscles exposed to diamide was 19 % less than muscles not exposed to diamide (Figure 2.3B). The greater decline in contractile force was associated with a 43 % higher mean total protein thiol oxidation level (Figure 2.3C), but no change in mean protein carbonylation level (Figure 2.3D). There was no change in total
protein thiols (Figure 2.3E). Overall, these findings are consistent with the hypothesis that protein thiol oxidation, but not protein carbonylation, affects muscle contractile force during fatiguing stimulation.

Figure 2.3. The effect of protein thiol modification on fatiguing stimulation and protein oxidation. Isolated EDL muscles were fatigued by stimulating at 50 Hz for 500 ms with a 15 s interval between contractions for a
period of 10 min while exposed to either 5 mM DTT or 1 mM diamide. Following 10 min of fatiguing stimulation, initial absolute force (A), mean percentage of initial force (B), percentage of oxidised protein thiols (C), protein carbonyls (D) and total protein thiols (E) were measured. *Significantly different measured at p<0.05 relative to fatigued muscles not exposed to either DTT or diamide. n=6. Values are expressed as means ± SE.

2.3.4 Evidence that oxidation of contractile proteins correlates with the loss of contractile force

In order to investigate whether oxidation of contractile proteins may be contributing to the loss in muscle contractile force during 10 min of fatiguing stimulation, some muscles were exposed to 10 mM caffeine at the fifth min of the 10 min fatiguing stimulation. There was only a partial recovery in contractile force to 65.3 ± 1.6 % from 46.0 ± 2.7 % (n=6, P<0.05) of the contractile force following the 10 min fatiguing stimulation. Since caffeine facilitated sarcoplasmic reticulum Ca\(^{2+}\) channel opening (Rousseau et. al, 1988), fatiguing effects associated with calcium availability would have been negated. Consequently, the lack of complete recovery in contractile force was consistent with the concept that oxidation of contractile proteins contributed to the loss of contractile force during fatiguing stimulation. However, the duration of caffeine exposure might be insufficient for caffeine to diffuse and reach steady state near fibers located in the muscle core center because large muscles were used here.

Skinned muscle fibers were also used as a model to further examine whether oxidative modification of contractile proteins affected muscle contractile force, independent of the effects on other cellular components such as the sarcoplasmic reticulum, cytosol and
mitochondria. This model was adopted because skinned muscle fibers allow solutes access to the intracellular space without the potential confounding effects caused by other non-contractile proteins involved in muscle contraction, such as Ca\(^{2+}\) handling proteins (Gutierrez-Martin et al., 2004; Aracena-Parks et al., 2006; Zissimopoulos et al., 2007).

In response to treatment with a protein thiol oxidising agent, diamide, there was a 29% decrease in maximal contractile force generated in skinned fibers (Figure 2.4A). When skinned fibers were subsequently exposed to the thiol reducing agent DTT, the contractile force generated by the fibers previously exposed to diamide recovered to 92% of control values (Figure 2.4A). The effect of exposing skinned muscle fibers to conditions which increases protein carbonylation level on muscle fiber contractile force were also examined. When skinned fibers were exposed to an oxidising reagent, FHA, which can cause protein carbonylation, there was a 32% decrease in contractile force (Figure 2.4B). However, when skinned fibers were subsequently exposed to DTT, there was no recovery in contractile force (Figure 2.4B).

Overall, these data demonstrate that oxidative stress can affect contractile force by either reversible protein thiol oxidation or irreversible protein oxidation of contractile proteins. Of note is that the effects of reversible protein thiol oxidation could be distinguished from the effects of irreversible oxidative protein damage by using the thiol reducing agent, DTT.
Figure 2.4. The effect of protein thiol oxidation and oxidative damage on contractile force of skinned muscle fibers. Skinned fibers were stimulated to contract after exposure to either 5 mM diamide (A) or 4 mM FeSO₄, 10 mM H₂O₂ and 25 mM ascorbate (FHA, B). Following contraction, skinned fibers were exposed to 1 mM DTT and stimulated to contract again (A, B). Mean percentage of control force were measured after exposure to different treatments. *Significantly different measured at p<0.05 relative to control skinned fibers not exposed to either diamide, FHA or DTT. n=8. Values are expressed as means ± SE.

2.3.5 Thiol oxidation of contractile proteins and the loss of contractile force

To investigate the effect of muscle stimulation on the thiol oxidation of contractile proteins, thiol oxidation levels of four major contractile proteins (actin, myosin, troponin and tropomyosin) were measured. Following 10 min of fatiguing stimulation, mean thiol oxidation levels of actin, myosin, troponin and tropomyosin increased by 22 % (Figure 2.5A), 55 % (Figure 2.5B), 34 % (Figure 2.5C) and 29 % (Figure 2.5D), respectively. There were no significant changes in mean actin (Figure 2.5A), troponin (Figure 2.5C) and tropomyosin (Figure 2.5D) thiol oxidation levels after 10 min of sustained stimulation. However, there was a 29 % (Figure 2.5B) increase in mean myosin thiol oxidation level after 10 min of sustained stimulation which was associated with a 5 % loss in contractile
force (Figure 2.1A). These data are consistent with the thiol oxidation of contractile proteins contributing to a loss of muscle contractile force in response to 10 min of fatiguing stimulation.

Figure 2.5. The effect of fatiguing and sustained stimulation on thiol oxidation of individual proteins.

Percentage thiol oxidation of actin (A), myosin (B), troponin (C) and tropomyosin (D) were measured after isolated EDL muscles were stimulated at 50 Hz for 500 ms with either a 15 s (fatigue) or 60 s (sustained) interval between contractions for a period of 10 min. *Significantly different measured at p<0.05 relative to resting muscles incubated for 10min without stimulation. n=6. Values are expressed as means ± SE.

If changes in the thiol oxidation level of a protein are responsible for changes in muscle contractile force, any change in thiol oxidation level should be accompanied by a change in contractile force. To investigate this further, the effect of DTT and diamide on specific
protein thiol oxidation and contractile force was examined. In this experiment, the improved contractile force after incubation in DTT was associated with 20 % (Figure 2.6A), 23 % (Figure 2.6B), 22 % (Figure 2.6C) and 23 % (Figure 2.6D) decrease in mean thiol oxidation levels of the actin, myosin, troponin and tropomyosin, respectively. The more pronounced declined contractile force after incubation in diamide was associated with 43 % (Figure 2.7A), 25 % (Figure 2.7B), 34 % (Figure 2.7C) and 26 % (Figure 2.7D) increase in mean thiol oxidation levels of the actin, myosin, troponin, and tropomyosin, respectively. There were significant correlations between the degree of actin ($R^2=0.557$, $P<0.05$), myosin ($R^2=0.290$, $P<0.05$), troponin ($R^2=0.460$, $P<0.05$), and tropomyosin ($R^2=0.388$, $P<0.05$) thiol oxidation and muscle contractile force (Figure 2.8) suggesting that contractile force decreases with the increase in thiol oxidation levels of contractile proteins.
Figure 2.6. The effect of protein thiol modification on thiol oxidation of individual proteins. Percentage thiol oxidation of actin (A), myosin (B), troponin (C) and tropomyosin (D) were measured after isolated EDL muscles were fatigued by stimulating at 50 Hz for 500 ms with a 15 s interval between contractions for a period of 10 min while exposed to either 5 mM DTT or 1 mM diamide. *Significantly different measured at p<0.05 relative to fatigued muscles not exposed to either DTT or diamide. n=6. Values are expressed as means ± SE.
To further examine the relationship between the thiol oxidation of the contractile proteins and contractile force, the four major contractile proteins were measured after 60 min of rest following the 10 min fatiguing stimulation. The recovery in contractile force was associated with recovery in the mean thiol oxidation levels of actin (Figure 2.8A), myosin (Figure 2.8B), troponin (Figure 2.8C) and tropomyosin (Figure 2.8D). However, only actin (Figure 2.8A) and myosin (Figure 2.8B) were associated with a full recovery. When muscles were stimulated again with the second bout of fatiguing stimulation, the decrease in mean thiol oxidation levels of the actin, myosin, troponin and tropomyosin were comparable.
with the effect of the first 10 min fatiguing stimulation (Figure 2.8A, B, C, D). Taken together, these data are consistent with thiol oxidation of actin and myosin contributing to the loss in muscle contractile force.

Figure 2.8. The effect of recovery from fatiguing stimulation on thiol oxidation of individual proteins. Percentage thiol oxidation of actin (A), myosin (B), troponin (C) and tropomyosin (D) were measured before first fatigue (rest), after first fatigue, after recovery and after second fatigue. *Significantly different measured at p<0.05. n=6. Values are expressed as means ± SE.
2.4 DISCUSSION

Although there is considerable evidence that muscle fatigue is mediated, at least in part, by increased oxidative stress, it is still unclear to what extent the effect of oxidative stress on muscle contractile performance is contributed by an increase in protein thiol oxidation or protein carbonylation levels. Using the EDL muscle preparation as the experimental model, this study shows that although fatiguing muscle stimulation increases protein carbonylation level and thus the degree of oxidative protein damage, it is protein thiol oxidation rather than protein carbonylation which is most likely to contribute to the ROS-mediated muscle fatigue associated with submaximal muscle stimulation in vitro. This study also shows for the first time that the thiol oxidation of several muscle contractile proteins (actin, myosin, tropomin and tropomyosin) increases in response to fatiguing muscle stimulation.

The results of this study are consistent with earlier studies which have provided evidence of increased oxidative protein damage (as measured by the carbonyl assay) following fatiguing stimulation (Figure 2.1C). Indeed, increased oxidative protein damage has been shown to occur in response to exercise (Reznick et al., 1992; Saxton et al., 1994; Radák et al., 1998; Barrerio et al., 2006; Pinho et al., 2006; Matsunaga et al., 2008; Veskoukis et al., 2008; Dubersteina et al., 2009; Agten et al., 2011). Similarly, increased oxidative damage to muscle proteins has also been reported in response to muscle contraction in vitro (Dalle-Donne et al., 2001; Canton et al., 2006; Coirault et al., 2007; Dalle-Donne et al., 2007; Fedorova et al., 2010; Shao et al., 2010). Despite the association between the rise in
protein carbonylation level and muscle fatigue, the results of this study show that elevated protein carbonylation and thus oxidative protein damage can occur without causing substantial muscle fatigue. Two observations support this contention. First, muscle contraction in the presence of the thiol reducing agent or thiol oxidising agent decreased and increased muscle fatigue, respectively, without affecting protein carbonylation level (Figure 2.3D). Second, the recovery of muscle contractile force following fatiguing stimulation was not associated with any fall in protein carbonylation level (Figure 2.2D). However, these results do not necessarily imply that the fall in muscle contractile performance during exercise cannot involve increased oxidative protein damage. Data from EDL muscles and skinned muscle fibers experiments showed that the use of an oxidative damage reagent increased protein carbonylation levels to approximately 3.5 nmol.mg\textsuperscript{-1} protein, and this cause a significant decrease in contractile force (Figure 2.4B), as opposed to the lower increase in protein carbonylation levels to approximately 2.3 nmol.mg\textsuperscript{-1} protein (Figure 2.1C) observed in the EDL muscles subjected to fatiguing stimulation.

This study provides further evidence that reversible protein thiol oxidation contributes towards the impairment in contractile performance during muscle fatigue. First, total protein thiol oxidation level increased in response to fatiguing stimulation, but not following sustained non-fatiguing stimulation (Figure 2.1B). Second, unlike protein carbonylation responses, the recovery of muscle contractile performance after fatiguing stimulation was accompanied by a return of total protein thiol oxidation to pre-stimulation level (Figure 2.2C). Third, both muscle fatigue and total protein thiol oxidation
level were more pronounced when fatiguing muscle stimulation took place in the presence of the thiol oxidising agent, diamide (Figure 2.3C). Fourth, muscle contraction performed with the thiol reducing agent, DTT, decreased the magnitude of both muscle fatigue and total protein thiol oxidation level (Figure 2.3C). These latter findings are consistent with earlier reports that the non-specific reducing and oxidising agents capable of affecting the level of protein thiol oxidation also affect muscle fatigue (Reid, 2008).

The novel finding that fatiguing muscle stimulation is associated with an increase in the thiol oxidation levels of four contractile proteins (actin, myosin, troponin and tropomyosin) is of interest as the functions of these proteins are known to be sensitive to oxidative stress. Indeed, previous work has shown that the thiol oxidation of actin (Hinshaw et al., 1991; Milzani et al., 2000; Dalle-Donne et al., 2003b; Hertelendi et al., 2008; Prochniewicz et al., 2008b), myosin (Brooke & Kaiser, 1970; Ajtai & Burghardt, 1989; Root & Reisler, 1992; Tiago et al., 2006; Hertelendi et al., 2008; Prochniewicz et al., 2008a), troponin (Putkey et al., 1993; Sousa et al., 2006; Pinto et al., 2008; Pinto et al., 2011), and tropomyosin (Williams & Swenson, 1982) affects their function. However, this study is the first one to show that muscle contraction to fatigue increases the thiol oxidation levels of these proteins, thus implying that muscle fatigue caused by oxidative stress is unlikely to be a consequence of the thiol oxidation of a single protein. It should be noted that thiol oxidation of other proteins could also be contributing to muscle fatigue in this study. For example, the function of SR Ca^{2+} ATPase and the SR Ca^{2+} release channel are sensitive to oxidative stress (Scherer & Deamer 1986; Abramanso...
Aracena-Parks et al., 2006; Zissimopoulos et al., 2007). Overall, although changes in the thiol oxidation of several proteins might contribute to fatigue, the extent to which each of these proteins contributes to muscle fatigue remains to be established.

Despite the evidence that the reversible rise in the thiol oxidation of muscle proteins contributes to muscle fatigue, it is estimated that only 20% of the overall loss of contractile force during fatiguing stimulation was from the contribution of protein thiol oxidation. This is suggested by the observation that although the addition of DTT returned the total protein thiol oxidation of fatigued muscles to levels comparable to those of the resting muscles, it improved contractile force by 20% during fatiguing stimulation. This calculation assumes that the extent to which all individual muscle proteins are thiol oxidised following fatiguing stimulation in the presence of DTT was comparable to resting muscles. This appears to be a reasonable assumption as the thiol oxidation of the contractile proteins (actin, myosin, troponin and tropomyosin) was comparable between DTT-treated fatigued muscles and resting muscles. These findings thus indicate that other factors such as the depletion of muscle glycogen stores (Ortenblad et al., 2011) and increased inorganic phosphate levels (Westerblad et al., 2002) play a more important role in mediating muscle fatigue under the experimental conditions adopted here.

In conclusion, this study corroborates for the first time, the view that oxidative stress contributes to muscle fatigue through protein thiol oxidation, and that oxidative protein damage does not necessarily contribute to muscle fatigue. The relationship between muscle fatigue and protein thiol oxidation appears to be complex as muscle fatigue was
associated with an increase in the thiol oxidation level of four contractile proteins (actin, myosin, troponin and tropomyosin). This raises the interesting possibility that the thiol oxidation of multiple proteins directly, or indirectly, associated with contractile force, contribute to ROS-mediated muscle fatigue.
CHAPTER 3:

PROTEIN THIOL OXIDATION AND

MUSCLE CONTRACTILE PERFORMANCE

AT 34 °C
ABSTRACT

The aim of this study was to examine whether protein thiol oxidation plays a role in ROS-mediated muscle fatigue at 34 °C, and whether the exposure of protein thiol reducing agent, dithiothreitol (DTT), during fatiguing stimulation at 34 °C affects protein thiol oxidation and muscle fatigue. Isolated extensor digitorum longus (EDL) muscles from male ARC(s) mice were incubated in Kreb’s Ringer solution at 34 °C and subjected to either a 10 min or 3.5 min fatiguing stimulation protocol consisting of 500 ms stimulation trains of 50 Hz at 15 s intervals. These muscles were compared to either muscles in a rested state or muscles stimulated to contract while exposed to DTT. Incubation of the EDL muscles at 34 °C with no contraction was associated with 14.0 ± 0.3 % (n=6) resting mean total protein thiol oxidation level, which was significantly higher than the 8.0 ± 0.2 % (n=6) found in vivo. In response to 10 min of fatiguing stimulation, contractile force fell by 51.3 ± 1.2 % (p<0.05), and this was associated with a 10.8 ± 5.1 % (p<0.05) higher mean total protein thiol oxidation level and a 20.2 ± 3.8 % (p<0.05) higher myosin mean thiol oxidation level compared to resting muscles. In response to 3.5 min of fatiguing stimulation, the force generated by muscles exposed to DTT increased progressively with time and was 87.4 ± 7.8 % (p<0.05) higher compared to stimulated muscles not exposed to DTT. The improvement in contractility was associated with a 27.3 ± 2.8 % (p<0.05) lower mean total protein oxidation and 22 ± 0.8 % (p<0.05), 25% ± 3.2 % (p<0.05), 12% ± 2.2 % (p<0.05) and 16% ± 3.2 % (p<0.05) lower actin, myosin, troponin and tropomyosin mean thiol oxidation levels, respectively, in DTT-treated muscles than DTT-untreated muscles. In conclusion, the effects of DTT on muscle contractile performance are consistent with muscle
contractile force being highly responsive to changes in the thiol oxidation of muscle proteins at physiological temperature, with proteins such as myosin and maybe actin, troponin and tropomyosin likely to be involved. Since the thiol oxidation of muscle proteins in muscle preparations at rest is well above normal, this suggests that DTT is beneficial to muscle contractile performance in part because it normalises muscle function rather than reduces muscle fatigue *per se*.

**Keywords:** Reactive oxygen species, protein thiols, dithiothreitol, muscle fatigue, 34 °C
3.1 INTRODUCTION

Reactive oxygen species (ROS) have been shown to be involved in the development of muscle fatigue (Ferreira et al., 2009; Kelly et al., 2009; Reardon & Allen, 2009a; Reardon & Allen, 2009b; Agten et al., 2011). One mechanism whereby ROS might contribute to muscle fatigue involves the reversible thiol oxidation of muscle proteins. The thiol moiety (-SH) of the cysteine residues of proteins can undergo reversible covalent reactions with ROS to form disulfide bonds (Eaton, 2006), which in turn can alter protein structure and function. Since muscle fatigue is also a reversible process, the reversible thiol oxidation of muscle proteins is generally considered to explain how ROS contribute to fatigue (Ferreira & Reid, 2008). In this regard, numerous muscle proteins can undergo reversible thiol oxidation when incubated in vitro with ROS. For instance, the thiol oxidation of actin can disrupt the organisation of the actin filaments, leading to reductions in the force generating ability of the sarcomere (Milzani et al., 2000). Peroxynitrite-induced oxidation of myosin has been shown to inhibit myosin ATPase activity, thus potentially impairing the force generating transition during the actomyosin ATPase cycle (Tiago et al., 2006). The function of troponin is also altered when oxidised (Putkey et al., 1993). Finally, the thiol oxidation of tropomyosin affects the ATPase activity of the actomyosin complex (Williams & Swenson, 1982).

Although the thiol oxidation of muscle proteins can affect their structure and function, it is important to stress that the aforementioned effects of thiol oxidation on individual muscle proteins have been based mainly on studies performed on purified proteins, and that
none of the studies published so far has examined whether the thiol oxidation of muscle proteins actually does increase with muscle fatigue. The first direct evidence that ROS-mediated muscle fatigue might be contributed to by the reversible thiol oxidation of several muscle proteins including myosin, actin, troponin and tropomyosin was provided by the results from Chapter 2. However, this work suggested that the reversible thiol oxidation of muscle proteins plays only a moderate role in muscle fatigue since the normalisation of the thiol oxidation of muscle proteins with the protein thiol reducing agent, dithiothreitol (DTT) during fatiguing stimulation improved contractile force generation by only 20 % (Chapter 2). Nonetheless, one limitation with these findings is that they were based on experiments performed at 25 °C on isolated extensor digitorum longus (EDL) from rats. Since this temperature is markedly different from that of working muscles; which may start as low as 30 °C and increase to 38 °C and even up to 40.8 °C when heat exhaustion occurs, (Allen et al., 2008), and considering that the importance of other mechanisms of fatigue (e.g. acidosis) is temperature sensitive (Adams et al., 1991; Pate et al., 1995; Westerblad et al., 1997), this raises the question of the importance of the thiol oxidation of muscle proteins contributing to muscle fatigue at physiological muscle temperature. For this reason, the primary objective of this study was to examine the extent to which protein thiol oxidation contributes to muscle fatigue at 34 °C, and the exposure to DTT during fatiguing stimulation at 34 °C decreases the magnitude of both protein thiol oxidation and muscle fatigue.
3.2 MATERIALS AND METHODS

3.2.1 Animals

Isolated EDL muscle preparations from eight weeks old male ARC(s) mice (n=24) were adopted as the experimental model. EDL muscles from mice were used to avoid potential issues with oxygen delivery to the muscle core (Lui et al., 2010), an important issue as oxygen consumption increases with temperature. All mice used in this study were obtained from the Animal Resources Centre (Murdoch University, Western Australia) and their mean weight was 38.1 ± 0.59 g. All animals were kept at a constant room temperature ranging between 22 and 24 °C, with free access to food and water. All procedures described here have been approved by the Animal Ethics Committee of the University of Western Australia.

3.2.2 Intact muscle preparation

All mice were anaesthetised with an intraperitoneal injection of pentobarbital (65 mg/kg of body weight), and both EDL muscles were carefully excised. While the first muscle was being tested, the other was left intact inside the animals and removed once experimentation with the first muscle was completed (≈10 min). Using a braided surgical silk (Size 5), one end of the muscle tendon was attached to a dual mode force transducer-servomotor (1200A Intact Muscle Test System, Aurora Scientific Inc, Canada) and the tendon at the other end of the muscle was attached to an anchoring hook. The muscle preparation was then bathed in Kreb’s mammalian Ringer solution (KRS) (Composition:
NaCl, 121 mM; KCl, 5.4 mM; MgSO₄, 1.2 mM; NaHCO₃, 25 mM; HEPES, 5 mM; glucose, 11.5 mM; CaCl₂, 2.5 mM at pH 7.4) gassed with 5% CO₂ and 95% O₂ and maintained at 34 °C throughout the experiment. The muscle was then stimulated with single twitches to attain the optimum length producing the highest force possible before experimentation. Force responses were elicited using a 701B muscle stimulator (Aurora Scientific Inc, Canada). Next, maximal force responses were obtained in response to supramaximal stimulation pulses (pulse width: 0.3 ms) delivered at 150 Hz for 500 ms with a 2-min rest between each stimulation to stabilise the force responses. The muscles were then given a 10 min rest before the commencement of the stimulation. The two submaximal fatiguing stimulation protocols used in the experiments on mice were pre-determined in the preliminary experiments. They involved exposure to stimulation pulses delivered (80 V and 1000 mA) at 50 Hz for 500 ms with a 15 s interval between contractions for either 3.5 min or 10 min.

3.2.3 Fatiguing stimulation protocol

In order to study the effect of fatiguing stimulation on protein thiol oxidation at 34 °C, isolated muscles were subjected to a 10 min fatiguing stimulation. Measurement made in these muscles were compared with resting muscles at 34 °C, but not stimulated to contract. After 10 min of fatiguing stimulation or rest, all muscles were frozen using aluminium clamps pre-cooled in liquid nitrogen and stored at -80 °C. In order to study the effect of the protein thiol reducing agent, DTT on fatiguing stimulation and protein thiol oxidation at 34 °C, isolated muscles were maintained in KRS with 25 mM DTT (n=6; Sigma
Chemical, Australia) during the initial 10 min resting period and an additional 25 mM of DTT was added into the bath immediately before the commencement of the 3.5 min fatiguing stimulation to replace the oxidised DTT dose with a freshly reduced DTT. A 3.5 min fatiguing stimulation time was adopted because preliminary experiments showed that two thirds of the fall in contractile force took place within 3.5 min, with a slow rate of fall in contractile force afterwards. Measurements made in these muscles were compared with fatigued muscles not exposed to DTT. After 3.5 min of fatiguing stimulation, all muscles were immediately frozen using aluminum clamps pre-cooled in liquid nitrogen and stored at -80 °C until analysed. In addition, muscles maintained in KRS with and without DTT were also frozen before the commencement of fatiguing stimulation.

3.2.4 Assay of proteins thiol oxidation levels

Protein thiol oxidation levels were determined using a fluorescent two-tag labelling technique, which involved the sequential labelling of reduced and oxidised protein thiol groups using two separate fluorescent tags on the same protein sample (Armstrong et al., 2011). Briefly, muscles were homogenised on ice with 20 % (w/v) trichloroacetic acid (TCA) in acetone using an Ultra-turax (IKA, Germany) and subsequently stored at -20 °C. The protein pellets extracted from 20 % (w/v) TCA in acetone were re-suspended in 50 μl of sodium dodecyl sulphate (SDS) buffer (0.5 % (w/v) SDS and 0.5 M Tris pH 7.0) in the presence of 0.5 mM BODIPY FL-N-(2-aminoethyl) maleimide (FLm) in the dark. All samples were sonicated and incubated for 30 min at room temperature in the dark with continuous vortexing. After centrifugation, the labelled protein solution was precipitated
with cold ethanol to remove excess dye (FLm), and then re-dissolved in 50 μl of SDS buffer (pH 7.0). Samples were reduced with the addition of 4 mM tris (2-carboxy-ethyl) phosphine hydrochloride (TCEP) and incubated for 60 min at room temperature in the dark. After reduction, samples were labelled with 0.25 mM Texas red-C2-maleimide (TRm), and incubated for 30 min at room temperature in the dark. Ethanol precipitation and re-solubilisation in SDS buffer was repeated four times to remove excess un-reacted dye (TRm). Samples were re-solubilized in 100 μL of SDS buffer, and the intensity of the fluorescence was measured for both FLm and TRm (FLm: excitation = 485 nm, emission = 520 nm and TRm: excitation = 595 nm, emission = 610 nm). Ovalbumin pre-labelled with a known quantity of FLm and TRm was used as a standard to quantify labelled protein thiols. Protein concentration (mg/ml) was determined using the Bio-Rad DC Protein Assay. The percentage of protein thiol oxidation was expressed as the concentration of oxidised thiols (TRm) to total protein thiols (FLm and TRm).

3.2.5 Electrophoretic separation and assay of ‘2 Tag’-labeled protein

To determine the levels of thiol oxidation of individual protein bands in the muscle sample, polyacrylamide gel electrophoresis was performed on the fluorescently labelled samples, prepared as described above, using the BioRad Mini Protean III system (Armstrong et al., 2011). Gel and buffer compositions were based on those described previously (Kohn & Myburgh, 2006), with the exceptions that a 12 % polyacrylamide gel was used and 5 mM DTT was added to the top running buffer. Electrophoresis was carried out at 8 mA with voltage not exceeding 150 V for 8.5 hr at 4 °C, with the buffer in the
lower chamber stirred with a magnetic stirrer throughout. Upon completion of the gel electrophoretic separation of proteins, the gel was immediately scanned for fluorescence using a Typhoon Scanner (Ammersham) at the wavelengths associated with both dyes (FLm: excitation = 485 nm, emission = 520 nm and TRm: excitation = 595 nm, emission = 610 nm). The individual protein bands corresponding to actin, myosin, tropomyosin and troponin were identified at molecular weight of these proteins of 42 kD, 205 kD, 33 kD and 18 kD respectively.

3.2.6 Statistical analysis

Results are presented as means ± standard error of the mean. All analyses were performed using either a Student’s paired t test or a one way ANOVA followed by Fisher LSD posteriori test, with significance at P<0.05. Sample sized is denoted by n.
3.3 RESULTS

3.3.1 The effect of fatiguing stimulation on protein thiol oxidation at 34 °C

To establish whether protein thiol oxidation increased in response to fatiguing muscle stimulation at a physiological temperature, isolated EDL muscles were subjected to a fatiguing stimulation for 10 min at 34 °C. Mean contractile force fell by approximately 51% (Figure 3.1A) following the fatiguing stimulation, and this was associated with an 11% increase in mean total protein thiol oxidation level (Figure 3.1B). There was no difference in total protein thiols (Figure 3.1C), indicating that the increase in protein thiol oxidation level likely represents increased oxidation of reduced protein thiols to oxidised protein disulfides. Incubation of the EDL muscles at 34 °C with no stimulation was associated with resting mean total protein thiol oxidation level of 14.0 ± 0.3 % (n=6) which was significantly higher than the in vivo mean total protein thiol oxidation level of 8.0 ± 0.2 % (n=6).
Figure 3.1. The effect of fatiguing stimulation on protein thiol oxidation at 34 °C. Isolated EDL muscles were fatigued by stimulating at 50 Hz for 500 ms with a 15 s interval between contractions for a period of 10 min. Following 10 min of stimulation, mean percentage of initial force (A), percentage of oxidised protein thiols (B) and total protein thiols (C) were measured. *Significantly different measured at p<0.05 relative to resting muscle incubated for 10 min without stimulation. n=6. Values are expressed as means ± SE.

3.3.2 The effect of the protein thiol reducing agent, DTT on fatiguing stimulation and protein thiol oxidation at 34 °C

In order to provide further evidence that protein thiol oxidation contributed to the loss in contractile force at 34 °C, fatiguing stimulation was also performed in the presence or absence of the protein thiol reducing agent, DTT. Following 3.5 min of fatiguing stimulation in the absence of DTT, the final contractile force of fatigued muscles
decreased to 66 % of the initial mean contractile force (Figure 3.2B). The mean total protein thiol oxidation level of fatigued muscles not exposed to DTT was 15 % higher than pre-fatigue level (Figure 3.2C). In contrast, fatiguing stimulation for 3.5 min in the presence of DTT increased the final contractile force of fatigued muscles to 124 % of the initial contractile force (Figure 3.2B). The mean total protein thiol oxidation level of fatigued muscles exposed to DTT was 12 % lower than the pre-fatigue level (Figure 3.2C). The initial 10 min incubation with DTT did not affect pre-fatigue mean total protein thiol oxidation level (Figure 3.2C). However, the effect of adding the second dose of DTT before the start of fatiguing stimulation on contractile force was evident after 0.5 min (Figure 3.2A).

The 87 % improvement in contractile force in fatigued muscles exposed to DTT was associated with a 27 % lower mean total protein thiol oxidation level than the fatigued muscles not exposed to DTT (Figure 3.2C). Given there was no difference in total protein thiols (Figure 3.2D), the decrease in protein thiol oxidation likely represents decreased oxidation of reduced protein thiols to oxidised protein thiols. These data therefore, show that although incubation with DTT did not affect pre-fatigue protein thiol oxidation level, exposure of DTT during fatiguing stimulation prevented the loss in contractile force, a finding consistent with protein thiol oxidation affecting muscle contractile force.
Figure 3.2. The effect of protein thiol reducing agent, DTT on fatiguing stimulation and protein thiol oxidation at 34 °C. Isolated EDL muscles were fatigued by stimulating at 50 Hz for 500 ms with a 15 s interval between contractions while exposed to 2 doses of 25 mM DTT for a period of 10 min before and at the start of stimulation or not exposed to DTT. Mean percentage of initial force (B), percentage of oxidised protein thiols (C) and total protein thiols (D) were measured at the start of fatigue in the absence or presence of DTT and after fatigue in the absence and presence of DTT. Percentage of mean force relative to initial force of fatigue in the absence of DTT (□) and fatigue in the presence of DTT (▲) were also measured every 0.5 min during the 3.5 min fatiguing stimulation (A). *Significantly different measured at p<0.05 relative to fatigued muscles not exposed to DTT. n=6. Values are expressed as means ± SE.
3.3.3  *Thiol oxidation of contractile proteins and the loss of contractile force at 34 °C*

In order to determine whether the thiol oxidation of individual contractile proteins might contribute to the loss of contractile force during the 10 min fatiguing stimulation at 34 °C, thiol oxidation levels were examined in four important contractile proteins (actin, myosin, troponin and tropomyosin). Following 10 min of fatiguing stimulation, the mean thiol oxidation level of myosin was 20% higher (Figure 3.3B), with no change in actin, troponin and tropomyosin mean thiol oxidation levels (Figure 3.3A, C, D). These data are consistent with the thiol oxidation of myosin contributing to the loss of contractile force during the 10 min of fatiguing stimulation at 34 °C.
Figure 3.3. The effect of fatiguing stimulation on thiol oxidation of individual proteins at 34 °C. Percentage thiol oxidation of actin (A), myosin (B), troponin (C) and tropomyosin (D) were measured after isolated EDL muscles were stimulated to fatigue at 50 Hz for 500 ms with a 15 s interval between contractions for a period of 10 min. *Significantly different measured at p<0.05 relative to resting muscles incubated for 10 min without stimulation. n=6. Values are expressed as means ± SE.

An investigation of whether thiol oxidation of individual contractile proteins could be affected by exposure to DTT during the 3.5 min fatiguing stimulation at 34 °C was carried out. The mean thiol oxidation levels of actin, myosin, troponin and tropomyosin were 58 % (Figure 3.4A), 37 % (Figure 3.4B), 36 % (Figure 3.4C) and 33 % (Figure 3.4D) higher, respectively, in muscles incubated at 34 °C relative to in vivo muscle.
Following 3.5 min of fatiguing stimulation in the absence of DTT, mean thiol oxidation levels of actin and myosin were 7% (Figure 4A) and 13% (Figure 3.4B) higher, respectively compared to pre-fatigue levels. In contrast, fatiguing stimulation for 3.5 min in the presence of DTT was associated with a 15% (Figure 3.4A), 11% (Figure 3.4B), 8% (Figure 3.4C) and 10% (Figure 3.4D) lower mean thiol oxidation levels of actin, myosin, troponin and tropomyosin, respectively, compared to pre-fatigue levels.

The 87% improvement in contractile force of fatigued muscles exposed to DTT was associated with a 22% (Figure 3.4A), 25% (Figure 3.4B), 12% (Figure 3.4C) and 16% (Figure 3.4D) decrease in mean actin, myosin, troponin and tropomyosin thiol oxidation levels, respectively compared to fatigued muscles not exposed to DTT. Taken together, these data suggest that increased protein thiol oxidation and thiol oxidation of myosin contributed to the loss in contractile force during fatigue at 34°C.
Figure 3.4. The effect of protein thiol reducing agent, DTT on thiol oxidation of individual proteins at 34°C.

Percentage thiol oxidation of actin (A), myosin (B), troponin (C) and tropomyosin (D) were measured in vivo, before the start of fatigue, after fatigue in the absence of DTT and after fatigue in the presence of DTT.

*Significantly different measured at p<0.05. n=6. Values are expressed as means ± SE.
3.4 DISCUSSION

The first direct evidence that ROS-mediated muscle fatigue associated with submaximal contraction \textit{in vitro} is mediated, in part, by the reversible thiol oxidation of muscle contractile proteins was provided in Chapter 2. However, muscle contraction performed in the presence of thiol reducing agent, DTT at levels that normalised the thiol oxidation of muscle proteins improved muscle contractile performance only to a moderate extent (Chapter 2), suggesting that the reversible thiol oxidation of contractile proteins plays only a moderate role in muscle fatigue. Since these findings were based on experiments performed at 25 °C rather than at a more physiological temperature, and that other mechanisms of muscle fatigue, such as those associated with acidosis, have been reported to play little role at physiological temperature (Adams et al., 1991; Pate et al., 1995; Westerblad et al., 1997). The issue of whether reversible protein thiol oxidation is an important contributor of muscle fatigue at physiological temperature was raised.

Using EDL muscle preparations from mice as the experimental model, this study shows that high levels of DTT not only prevents a fall in the contractile force of the muscles tested at physiological temperature, but actually results in a progressive increase in muscle contractile force, reaching 87 % more than that attained in fatigued muscles not exposed to DTT (Figure 3.2B). Since the intensity of the contraction protocol was submaximal, it is unclear whether the large DTT-mediated improvement in contractile force resulted from an increase in maximal force or a shift in the force-frequency toward higher frequencies due to decreased fusion of twitch (or individual) contractions. The
marked benefit of such DTT treatment on muscle contractile performance was associated with the absence of a rise in the total thiol oxidation of muscle proteins (Figure 3.2C), including myosin, actin, tropomyosin and troponin (Figure 3.4) compared to no DTT treatment. However, since the thiol oxidation of muscle proteins were above normal in resting muscles at 34 °C compared to in vivo muscles (freshed dissected), these findings do not allow one to evaluate the importance of protein thiol oxidation in ROS-mediated muscle fatigue since part of the effect of DTT treatment is to normalise muscle function rather than preventing muscle fatigue. Nevertheless, irrespective of the extent to which DTT affects muscle fatigue, this is the first study to show that changes in the thiol oxidation of muscle proteins can be associated with major effects on muscle contractile performance at 34 °C.

The findings of this study suggest that muscle contractile force at physiological temperature is highly sensitive to the thiol oxidation of muscle proteins. Firstly, this is supported by the observation that total protein thiol oxidation level increased in response to fatiguing stimulation (Figure 3.1B). Secondly, acute exposure to the thiol reducing agent, DTT improved muscle contractile force with time (Figure 3.1A) while decreasing the thiol oxidation levels of muscle proteins including that of myosin, actin, troponin and tropomyosin (Figure 3.4). These results not only corroborate earlier findings from Chapter 2, that reversible protein thiol oxidation affects muscle contractile force (Chapter 2), but also are consistent with the many earlier reports that nonspecific reducing agents can affect muscle contractile performance (Reid, 2008). It is important to stress, however, that although many of these studies have implicated oxidative stress as a mediator of muscle
contractile performance, none of these studies have examined whether reversible thiol oxidation of muscle proteins has the capacity to affect muscle contractile force.

The marked increase in the thiol oxidation level of muscle proteins in response to fatiguing stimulation raises the obvious issue of the identity of the proteins involved. Close inspection of the responses of individual protein bands separated by gel electrophoresis suggests that the thiol oxidation of myosin might contribute to muscle fatigue in the in vitro model investigated here at a more physiological temperature, whereas the absence of changes in actin, troponin and tropomyosin thiol oxidation suggests that these proteins are not involved. However, since DTT treatment and associated improvement in muscle contractile performance were accompanied by a fall in the thiol oxidation levels of myosin, actin, troponin, and tropomyosin, it is possible that the thiol oxidation of actin, troponin and tropomyosin also affects muscle contractile force. This interpretation that myosin and other contractile proteins affect muscle contractile force is consistent with earlier reports that the thiol oxidation of myosin causes a marked decrease in myosin ATPase activity (Root & Reisler, 1992) due to a decrease in the fraction of myosin heads undergoing the force-generating transition step during the actomyosin ATPase cycle (Tiago et al., 2006). Others have also shown that changes in the thiol oxidation of actin, troponin and tropomyosin affect not only their structures, but also their functions (Putkey et al., 1993; Williams & Swenson, 1982; Milzani et al., 2000).

The benefits of DTT treatment on muscle contractile performance suggests that the thiol oxidation of muscle proteins might play some role in ROS-mediated muscle fatigue at
physiological temperature. However, a higher than normal thiol oxidation level of muscle proteins prior to contraction together with the progressive temporal increase in muscle contractile force exposed to DTT during stimulation suggests that DTT is beneficial, in part, because it normalises muscle function rather than only opposing muscle fatigue. This is likely the case here as many studies have reported an increased production of ROS with increased muscle temperature (Arbogast & Reid, 2004; Edwards et al., 2007), and a marked decrease in muscle performance above 35 °C (Segal et al., 1985, 1986; Pedersen et al., 2003). In addition, there is strong evidence that the generation of ROS explains the reduced longevity of \textit{in vitro} skeletal muscle preparations at temperatures ranging between 37-43 °C (Zuo et al., 2000; Edwards et al., 2007; van der Poel et al., 2002; Arbogast & Reid, 2004; Moopanar & Allen, 2005; van der Poel et al., 2007).

These findings thus highlight some of the limitations with using isolated muscle preparations as experimental models to investigate ROS-mediated muscle fatigue at physiological temperature due to the associated abnormal increase in protein thiol oxidation level compared to muscle \textit{in vivo}. Investigating muscle fatigue at a lower and unphysiological temperature is clearly not a suitable alternative despite being associated with normal protein thiol oxidation (Chapter 2). This is because of the possibility that the importance of some of the mechanisms of muscle fatigue is temperature sensitive. This is best illustrated by the finding that acidosis is an important mediator of fatigue in muscles incubated at room temperature, but only plays a marginal role at physiological temperature (Adams et al., 1991; Pate et al., 1995; Westerblad et al., 1997). Thus, future studies investigating the physiological importance of the thiol oxidation of muscle proteins
in muscle fatigue *in vitro* requires the introduction of better models that do not affect protein thiol oxidation at physiological temperature.

In conclusion, the effects of DTT on muscle contractile performance are consistent with muscle contractile force being highly responsive to changes in the thiol oxidation of muscle proteins at physiological temperature, with proteins such as myosin and maybe actin, troponin and tropomyosin likely to be involved. Since the thiol oxidation of muscle proteins in muscle preparations at rest is well above normal, these results at physiological temperature are consistent with the possibility that DTT is beneficial in part, to muscle contractile performance. This is because it normalises muscle function rather than reduces muscle fatigue *per se*, thus leaving without a definitive answer the importance of the role played by protein thiol oxidation in ROS-mediated muscle fatigue at physiological temperature. This chapter, however, again clearly shows for the first time that the thiol oxidation of muscle proteins is associated with large changes in muscle contractile performance. This raises the possibility that protein thiol oxidation has the potential to be an important mediator of ROS-mediated muscle fatigue, and is worth further investigation.
CHAPTER 4:

PROTEIN THIOL OXIDATION AND
MUSCLE CONTRACTILE FUNCTION IN
INS2AKITA DIABETIC MICE
ABSTRACT

The aim of this study was to examine whether the thiol oxidation level of muscle proteins is increased before and after muscle contraction in diabetic Ins2Akita mice, and whether this is associated with lower muscle contractile performance. Isolated extensor digitorum longus muscles from male Ins2Akita mice were subjected to a fatiguing stimulation that consisted of 10 min of submaximal tetanic contraction of 500 ms duration at 15 s intervals with a stimulation frequency of 50 Hz. These muscles were compared with muscles from non-diabetic mice subjected to the same fatiguing stimulation. Initial specific force did not differ between the different muscles; however the initial mean thiol oxidation levels of total proteins, actin and tropomyosin in muscles from diabetic mice compared to non-diabetic mice were 16.5 ± 0.2 % (p<0.05), 16.3 ± 0.4 % (p<0.05) and 7.6 ± 0.4 % (p<0.05) higher, respectively. Following 10 min of fatiguing stimulation, contractile force of muscles from diabetic mice fell by 27.4 ± 1.2 % (p<0.05) more than non-diabetic mice and this was associated with a 21.8 ± 0.3 % (p<0.05), 15.5 ± 0.5 % (p<0.05) and 18.9 ± 0.4 % (p<0.05) higher mean thiol oxidation of total proteins, actin, and tropomyosin in muscles from diabetic mice compared to non-diabetic mice, respectively. It is concluded that initial specific force of the muscles from diabetic mice compared to non-diabetic mice is not reduced despite higher total protein thiol oxidation in muscles from diabetic mice. However, the higher thiol oxidation level of myosin in muscles from diabetic mice exposed to fatiguing stimulation might explain the lesser resistance of these muscles to fatigue.

Keywords: Reactive oxygen species, protein thiols, Ins2Akita mice, muscle fatigue
4.1 INTRODUCTION

Regular exercise provides a number of well documented health benefits for individuals with Type 1 diabetes mellitus (T1DM) including improvements in cardiovascular function (Choi & Chisholm, 1996; Fuchsjager-Mayrl et al., 2002), blood lipid profile (Lehmann et al., 1997; Laaksonen et al., 2000), weight control (Lehmann et al., 1997) and quality of life (Zoppini et al., 2003). Unfortunately, people with T1DM have reduced cardiopulmonary fitness (Raev, 1994; Niranjan et al., 1997; Komatsu et al., 2005, Nadeau et al., 2010) compared to their healthy non-diabetic peers. This is unfortunate considering that premature cardiovascular disease significantly shortens the average lifespan of individuals with T1DM (Libby et al., 2005).

One factor that has the potential to limit the ability of T1DM individuals to engage in an active lifestyle compared to their peers is their reduced motor function. Diabetic peripheral neuropathies are one cause of this reduced motor function as isokinetic muscle strength in long-term T1DM patients is reduced to an extent that increases with the severity of neuropathy (Andersen et al., 1996; Andersen et al., 1997; Andreassen et al., 2006). Motor function is also reduced in neuropathy-free T1DM individuals (Andersen, 1998; Andersen et al., 2005; Almeida et al., 2008). These individuals have lower motor conduction velocities, and the onset of fatigue is much earlier than non-diabetic individuals (Almeida et al., 2008). Finally, young T1DM individuals have reduced resistance to skeletal muscle fatigue compared to non-diabetic individuals (Ramiere et al., 1993).
There is indirect evidence that the poor muscle contractile performance in diabetes might be related to the increase in oxidative stress associated with this disease (Nishikawa et al., 2001; Evans et al., 2005; Kaneki et al., 2007), a condition characterised by an imbalance between the production of reactive oxygen species (ROS; e.g. superoxide, hydroxyl radicals) and removal by antioxidant defences. For instance, marked increase in ROS production has also been reported during exercise and pharmacologic antioxidants has been shown to improve muscle fatigue (Reid, 2008). These evidences together with the results of Chapters 2 and 3 that changes in the thiol oxidation level of muscle proteins can acutely affect muscle contractile performance raise the question of whether impaired contractile performance associated with diabeties might be contributed, at least in part, by an increase in the thiol oxidation level of skeletal muscle proteins. Using isolated extensor digitorum longus (EDL) muscle preparations from diabetic Ins2Akita mice and wild type non-diabetic mice as controls, the primary objective of this study was to examine whether the thiol oxidation levels of muscle proteins was increased before and after muscle contraction in diabetic mice, and whether this was associated with lower muscle contractile performance.
4.2 MATERIALS AND METHODS

4.2.1 Animals

Male Ins2Akita mice (n=5) bred on a C57BL/6J background and non-diabetic C57BL/6J mice (n=8) at 40 weeks of age and non-diabetic C57BL/6J mice (n=10) at 13 weeks of age were housed under controlled temperature of 22 °C with a 12:12hr light/cycle (lights on at 0600hr), with food and water available ad libitum. Ins2Akita mice were used as they undergo mutation resulting in a single amino acid substitution in the insulin 2 gene, which causes misfolding of the insulin protein leading to significant hyperglycemia, as early as four weeks of age (Barber et al., 2005). All experiments were approved by the Animal Ethics Committee of the University of Western Australia.

4.2.2 Intact muscle preparation

Following a 12 hr overnight fast, mice were anaesthetised with an intraperitoneal injection of pentobarbital (65 mg/kg of body weight). Blood glucose and glycated haemoglobin levels (HbA1c) were then immediately measured using Accu-Check Performa Blood Glucose Meter (Roche, Sydney, Australia) and DCA 2000®+ Analyser (Siemens, USA), respectively. Next, both EDL muscles were carefully removed one after the other for experiments. While the first muscle was being tested, the other was left intact inside the animals and removed once experimentation with the first muscle was completed (≈10 min). After removing the muscles, one end of the muscle tendon was attached to a dual mode force transducer-servomotor (1200A Intact Muscle Test System, Aurora Scientific...
Inc, Canada) and the other tendon to an anchoring hook using a braided surgical silk (Size 5). The muscle preparation was bathed in Kreb’s mammalian Ringer solution (KRS) (Composition: NaCl, 121 mM; KCl, 5.4 mM; MgSO$_4$, 1.2 mM; NaHCO$_3$, 25 mM; HEPES, 5 mM; glucose, 11.5 mM; CaCl$_2$, 2.5 mM at pH 7.4) gassed with 5% CO$_2$ and 95% O$_2$ at 25 °C throughout the experiment. The muscle was then stimulated with single twitches to attain the optimum length producing the highest force possible before experimentation. Force responses were then elicited using a 701B muscle stimulator (Aurora Scientific Inc, Canada). Next, maximal force responses were obtained in response to supramaximal stimulation pulses (pulse width: 0.3 ms) delivered at 150 Hz for 500 ms with a 2 min rest between each stimulation to stabilise the force responses. The muscles were then given a 10-min rest before the commencement of the stimulation. The submaximal stimulation protocol used in the experiments on mice was pre-determined in the preliminary experiments. It involved exposure to stimulation pulses (80 V and 1000 mA) delivered at 50 Hz for 500 ms with a 15 s interval between contractions for 10 min.

4.2.3 Fatiguing stimulation protocol

For all experiments, isolated muscles were maintained for 10 min in KRS before stimulation. In order to study the effect of contraction on the protein thiol oxidation of skeletal muscles, isolated muscles from diabetic and non-diabetic mice were subjected to a 10 min fatiguing stimulation. Immediately after stimulation, all muscles were frozen using an aluminium clamp pre-cooled in liquid nitrogen and stored at -80 °C. In addition, resting EDL muscles obtained from the contralateral leg of each of the tested animals
were also frozen before the commencement of the fatiguing stimulation. In order to study the effect of protein thiol modification on muscle contraction and protein thiol oxidation, isolated muscles from non-diabetic mice were maintained for 10 min in KRS with 15 mM dithiothreitol (DTT) before being subjected to a single contraction that consisted of submaximal tetanic contraction of 500 ms duration at a stimulation frequency of 50 Hz. These muscles were compared with contracting muscles not exposed to DTT. After contraction, all muscles were frozen using aluminium clamps pre-cooled in liquid nitrogen and stored at -80 °C.

4.2.4 Assay of proteins thiol oxidation levels

Protein thiol oxidation levels were determined using a fluorescent two-tag labelling technique, which involved the sequential labelling of reduced and oxidised protein thiol groups using two separate fluorescent tags on the same protein sample (Armstrong et al., 2011). Briefly, muscles were homogenised on ice with 20 % (w/v) trichloroacetic acid (TCA) in acetone using an Ultra-turax (IKA, Germany) and subsequently stored at -20 °C. The protein pellets extracted from 20 % (w/v) TCA in acetone were re-suspended in 50 µl of sodium dodecyl sulphate (SDS) buffer (0.5 % (w/v) SDS and 0.5 M Tris pH 7.0) in the presence of 0.5 mM BODIPY FL-N-(2-aminoethyl) maleimide (FLm) in the dark. All samples were sonicated and incubated for 30 min at room temperature in the dark with continuous vortexing. After centrifugation, the labelled protein solution was precipitated with cold ethanol to remove excess dye (FLm), and then re-dissolved in 50 µl of SDS buffer (pH 7.0). Samples were reduced with the addition of 4 mM tris (2-carboxy-ethyl)
phosphine hydrochloride (TCEP) and incubated for 60 min at room temperature in the dark. After reduction, samples were labelled with 0.25 mM Texas red-C2-maleimide (TRm), and incubated for 30 min at room temperature in the dark. Ethanol precipitation and re-solubilisation in SDS buffer was repeated four times to remove excess un-reacted dye (TRm). Samples were re-solubilized in 100 μL of SDS buffer, and the intensity of the fluorescence was measured for both FLm and TRm (FLm: excitation = 485 nm, emission = 520 nm and TRm: excitation = 595 nm, emission = 610 nm). Ovalbumin pre-labelled with a known quantity of FLm and TRm was used as a standard to quantify labelled protein thiols. Protein concentration (mg/ml) was determined using the Bio-Rad DC Protein Assay. The percentage of protein thiol oxidation was expressed as the concentration of oxidised thiols (TRm) to total protein thiols (FLm and TRm).

4.2.5 Electrophoretic separation and assay of ‘2 Tag’-labeled protein

To determine the levels of thiol oxidation of individual protein bands in the muscle sample, polyacrylamide gel electrophoresis was performed on the fluorescently labelled samples, prepared as described above, using the BioRad Mini Protean III system (Armstrong et al., 2011). Gel and buffer compositions were based on those described previously (Kohn & Myburgh, 2006), with the exceptions that a 12 % polyacrylamide gel was used and 5 mM DTT was added to the top running buffer. Electrophoresis was carried out at 8 mA with voltage not exceeding 150 V for 8.5 hr at 4 °C, with the buffer in the lower chamber stirred with a magnetic stirrer throughout. Upon completion of the gel electrophoretic separation of proteins, the gel was immediately scanned for fluorescence
using a Typhoon Scanner (Ammersham) at the wavelengths associated with both dyes (FLm: excitation = 485 nm, emission = 520 nm and TRm: excitation = 595 nm, emission = 610 nm). The individual protein bands corresponding to actin, myosin, tropomyosin and troponin were identified at molecular weight of these proteins of 42 kD, 205 kD, 33 kD and 18 kD respectively.

4.2.6 Statistical analysis

Results are presented as means ± standard error of the mean. All analyses were performed using a Student’s unpaired t test or a two way ANOVA followed by Fisher LSD posteriori test, with significance at P<0.05. Sample sized is denoted by n.
4.3 RESULTS

4.3.1 Body mass, fasting plasma glucose level and fasting glycated hemoglobin of non-diabetic mice and diabetic mice

The mean body mass of non-diabetic mice was 55 % (Figure 4.1A) higher than that of diabetic mice. Mean fasting blood glucose levels and mean HbA1c levels were 137 % (Figure 4.1B) and 84 % (Figure 4.1C) higher, respectively, in the diabetic mice compared to the non-diabetic mice. These results indicate that the Ins2akita mice used in this study showed clear signs of diabetes.

Figure 4.1. Body mass, fasting plasma glucose level and fasting glycated hemoglobin of non-diabetic mice (CON) and diabetic mice (DM). Body mass (A), fasting plasma glucose level (B) and fasting glycated haemoglobin (C) in non-diabetic mice (n=8) and diabetic mice (n=5). *Significantly different measured at p<0.05. Values are expressed as means ± SE.
4.3.2 The relationship between protein thiol oxidation of non-diabetic mice and diabetic mice on associated muscle functions

Initial specific force at the start of fatiguing stimulation did not differ between the muscles from non-diabetic mice and diabetic mice (Figure 4.2A). Initial pre-fatigue mean total protein thiol oxidation level was 16 % higher in the muscles of diabetic mice than non-diabetic mice (Figure 4.2C). Following 10 min of fatiguing stimulation, contractile force of the muscles from diabetic mice fell by 27 % more than muscles from non-diabetic mice (Figure 4.2B), and mean total protein thiol oxidation levels were 10 % and 15 % higher in muscles of non-diabetic mice and diabetic mice, respectively (Figure 4.2C). The 27 % lower contractile force in muscles from diabetic mice was associated with a 22 % (Figure 4.2C) higher mean total protein thiol oxidation level. There was no difference in total protein thiols (Figure 4.2D) so the increase in protein thiol oxidation level likely represents increased conversion of reduced protein thiols to oxidised protein thiols.

The resting mean total protein thiol oxidation levels of the muscles from non-diabetic mice and diabetic mice incubated at 25 °C with no contraction were 15.7 ± 0.3 % (n=8) and 18.3 ± 0.3 % (n=5), which were significantly higher than 7.5 ± 0.2 % (n=5) and 10.7 ± 0.3 % (n=5) measured in in vivo muscles from non-diabetic mice and diabetic mice, respectively, frozen immediately after dissection, indicating that incubation resulted in an increase in protein thiol oxidation level.
Figure 4.2. The relationship between protein thiol oxidation of non-diabetic mice (CON) and diabetic mice (DM) on associated muscle functions. Isolated EDL muscles (CON, n=8; DM, n=5) were fatigued by stimulating at 50 Hz for 500 ms with a 15 s interval between contractions for a period of 10 min. At the start of the stimulation, initial specific force (A) was measured. Following 10 min of stimulation, mean percentage of initial force (B), percentage of protein thiols oxidised (C) and total protein thiols (D) were measured. *Significantly different measured at p<0.05 relative to pre-fatigue muscles (CON, n=8; DM, n=5). Values are expressed as means ± SE.

4.3.3 Thiol oxidation of contractile proteins and the loss of contractile force

In order to identify the proteins likely to be affected by muscle contraction to fatigue, muscle extracts labelled for protein thiol oxidation were subjected to gel electrophoresis
to examine the behavior of actin, myosin, troponin and tropomyosin. Immediately before
the start of fatiguing stimulation, the mean thiol oxidation levels of actin and tropomyosin
were 16 % (Figure 4.3A) and 8 % (Figure 4.3D) higher, respectively, in muscles from
diabetic mice than non-diabetic mice. There were no significant differences in the mean
thiol oxidation levels of myosin (Figure 4.3A) and troponin (Figure 4.3C) before the start of
fatiguing stimulation.

Following 10 min of fatiguing stimulation, the mean thiol oxidation levels of actin in
muscles from non-diabetic mice and diabetic mice were 11 % and 10 % (Figure 4.3A)
higher compared with pre-fatigue levels, respectively. The mean thiol oxidation levels
myosin in muscles from non-diabetic mice and diabetic mice were 13 % and 28 % (Figure
4.3B) higher compared with pre-fatigue levels, respectively. There were no significant
differences in the mean thiol oxidation levels of troponin (Figure 4.3C) and tropomyosin
(Figure 4.3D) following 10 min of fatiguing stimulation compared with pre-fatigue levels.

The 27 % lower contractile force in muscles from diabetic mice (Figure 4.2B) was
associated with 15 % (Figure 4.3A) and 19 % (Figure 4.3B) higher actin and myosin mean
thiol oxidation levels respectively compared to muscles from non-diabetic mice after the
fatiguing stimulation.
Figure 4.3. The effect of fatiguing stimulation on thiol oxidation of individual proteins in non-diabetic mice (CON) and diabetic mice (DM). Percentage thiol oxidation of actin (A), myosin (B), troponin (C) and tropomyosin (D) were measured before and after EDL muscles (CON, n=8; DM, n=5) were fatigue by stimulating at 50 Hz for 500 ms with a 15 s interval between contractions for a period of 10 min.

*Significantly different measured at p<0.05. Values are expressed as means ± SE.

4.3.4 The effect of protein thiol modification on muscle contraction and protein thiol oxidation

Given that muscle incubation in KRS for 10 min was associated with an increase in protein thiol oxidation level, the effect of the protein thiol reducing agent, DTT, on contractile
force was examined. To this end, EDL muscles from non-diabetic mice were titrated with different levels of DTT, and 15 mM of DTT was found to significantly increase the specific contractile force. The mean contractile force of muscles exposed to DTT was 48 % higher than those not exposed to antioxidant (Figure 4.4A). The improvement in contractile force was associated with a 34 % lower mean total protein thiol oxidation level (Figure 4.4B). There was no difference in total protein thiols (Figure 4.4C).

The stimulatory effect of DTT on muscle contraction was accompanied by a significant change in the thiol oxidation levels of the four major contractile proteins examined here. The improvement in contractile force of muscles incubated with DTT was associated with 33 % (Figure 4.5A), 33 % (Figure 4.5B), 31 % (Figure 4.5C) and 30 % (Figure 4.5D) decrease in the mean thiol oxidation levels of the actin, myosin, troponin and tropomyosin, respectively compared to muscles not exposed to with antioxidant.
Figure 4.4. The effect of protein thiol modification on single muscle contraction and protein thiol oxidation. Isolated EDL muscles from non-diabetic mice were stimulated once at 50 Hz for 500 ms while exposed to 15 mM DTT. Following the single contraction, specific force (A), percentage of oxidised protein thiols (B) and total protein thiols (C) were measured. *Significantly different measured at p<0.05 relative to contracting muscles not exposed to DTT. n=5. Values are expressed as means ± SE.
Figure 4.5. The effect of protein thiol modification on thiol oxidation of individual proteins. Percentage thiol oxidation of actin (A), myosin (B), troponin (C) and tropomyosin (D) were measured after isolated EDL muscles from non-diabetic mice were stimulated once at 50 Hz for 500 ms while exposed to 15 mM DTT. *Significantly different measured at p<0.05 relative to contracting muscles not exposed to DTT. N=5. Values are expressed as means ± SE.
4.4 DISCUSSION

This study examined whether the lower muscle strength and lower resistance to muscle fatigue associated with diabetes might be contributed, at least in part, by a higher than normal thiol oxidation level of muscle proteins. In order to determine whether this is the case, the thiol oxidation levels of muscle proteins and muscle contractile function were compared between diabetic Ins2Akita mice and wild type non-diabetic mice. Contrary to expectations, there was no difference in maximal specific force despite the higher total mean protein thiol oxidation in muscles from diabetic mice. However, muscles from diabetic mice were less resistant to fatigue, and had a higher mean total protein thiol oxidation level compared to muscles from non-diabetic mice.

The absence of a difference in initial specific force between the non-diabetic mice and diabetic mice (Figure 4.2A) does not support past findings that diabetes impairs muscle strength. It has been reported that isokinetic muscle strength is reduced in T1DM patients (Andersen et al., 1996; Andersen et al., 1997; Andreassen et al., 2006) as well as in other animal models of diabetes. In particular, insulin-untreated STZ-diabetes in rats results in a fall in the specific tension of the EDL (Cotter et al., 1989; Cotter et al., 1993; McGuire & MacDermott, 1999). Similarly, alloxan-induced diabetes reduces the tetanic force of the EDL (Paulus & Grossie, 1983). Finally, skinned-fibers from the EDL of STZ-diabetic rats have lower maximal specific tension compared to those of non-diabetic animals (Stephenson et al., 1994).
However, the absence of difference in initial specific force might be explained, at least in part, by the possible differences between muscle preparations from these mice. These preparations were exposed to similar physiological glucose concentrations, unlike the different glucose concentrations to which the muscles are exposed in the intact animals. Exposing the muscles from diabetic mice to the high glucose concentrations normally found in these mice would have possibly affected their contractile function compared to muscles from non-diabetic animals. This is suggested by the observation that acute hyperglycaemia in healthy individuals with T1DM is associated with lower maximal isometric voluntary contraction compared to euglycaemia (Andersen et al., 2005). It is important to stress, however, that some marked differences in muscle specific force were expected between diabetic and non-diabetic animals even when exposed to matching glucose concentrations because studies performed by others on isolated muscle preparation from rats reported impaired muscle tension in STZ-diabetic compared to non-diabetic animals despite being tested at identical glucose levels (Paulus & Grossie, 1983; Stephenson et al., 1994).

The finding that total protein thiol oxidation from diabetic mice was increased compared to non-diabetic mice (Figure 4.2C) is consistent with the observations of others that diabetes is associated with an increase in oxidative stress (Nishikawa et al., 2001; Evans et al., 2005; Kaneki et al., 2007). However, since the increase in total protein thiol oxidation was not associated with lower muscle maximal specific tension in muscles from diabetic mice compared to non-diabetic mice, this would appear not only to refute the hypothesis of a link between muscle protein thiol oxidation in diabetes and impaired muscle tension,
but would also suggest that muscle contractile performance is unresponsive to muscle protein thiol oxidation. This latter interpretation, however, is inconsistent with the findings in Chapters 2 and 3 which showed a link between muscle contractile force and the thiol oxidation of muscle proteins, and the findings here that muscle tension increases and the thiol oxidation levels of muscle proteins falls in the presence of DTT.

The observation that elevated total protein thiol oxidation level of muscles from diabetic mice was not accompanied by a fall in maximal specific force could be explained on the grounds that although total protein thiol oxidation level in the muscles of diabetic mice was higher than muscle of non-diabetic mice, this might not be the case at the level of specific proteins known to affect muscle contractile function. That this might be the case here, is suggested by the findings in pre-fatigue muscles that although the thiol oxidation levels of actin and tropomyosin were higher in fatigued muscles of diabetic mice (Figure 4.3A,D), no differences were detected in the thiol oxidation levels of myosin and troponin (Figure 4.3B,C) in pre-fatigue and fatigued muscles of diabetic mice. Given the role played by myosin in muscle contraction and previous findings that the oxidation of myosin inhibits myosin ATPase activity thus potentially impairing the force generating transition during the actomyosin ATPase cycle (Tiago et al., 2006), the absence of a difference in the thiol oxidation levels of myosin and other contractile proteins between muscles from non-diabetic mice and diabetic mice might explain their similar maximal specific force. Arguably, this interpretation holds as long as myosin plays a more important role in determining contractile force compared with other contractile proteins such as actin, troponin and tropomyosin. Although further work is required to test this interpretation,
the findings with myosin highlight the importance of examining the response to individual proteins compared to total proteins.

The finding that resistance to muscle fatigue is lower in muscles in diabetic mice (Figure 4.2B) concurs with experiments performed in T1DM individuals. Non-diabetic individuals are more resistant to fatigue than T1DM individuals, particularly those in poor glycaemic control (Ramiere et al., 1993; Almeida et al., 2008). This is also the case in T1DM individuals free of diabetic complications (Ramiere et al., 1993). However, this is not the case in animal models of diabetes, resistance to fatigue is not affected by STZ-induced diabetes in the EDL muscle of insulin-untreated STZ-diabetic rats although muscle specific tension is impaired as mentioned above (Cotter et al., 1989; Cotter et al., 1993; McGuire & MacDermott, 1999). The greater fall in force in muscles from diabetic mice could be attributed to their higher total protein thiol oxidation level compared to muscles from non-diabetic mice. However, one difficulty with this interpretation is that the total protein thiol oxidation level of muscle proteins attained after fatiguing stimulation in muscles from non-diabetic mice were comparable to those of diabetic mice prior to contraction (Figure 4.2C). This suggests that changes in protein thiol oxidation might not play an important role in fatigue.

A different interpretation arises when the thiol oxidation levels of individual proteins are compared between muscles from non-diabetic mice and diabetic mice. Although the thiol oxidation levels of actin, troponin and tropomyosin did not differ between fatigued muscles from non-diabetic mice and pre-fatigue muscles from diabetic mice (Figure
4.3A,C,D), the thiol oxidation level of myosin was lower in pre-fatigue muscles from diabetic mice compared to fatigued muscles from non-diabetic mice (Figure 4.3B; p<0.05). If the thiol oxidation of myosin was to play a major role in muscle force generation and fatigue, this observation alone would explain why resistance to muscle fatigue is lower in muscles from diabetic mice compared to muscles from non-diabetic mice despite comparable protein thiol oxidation levels. However, a cautionary approach should be adopted with this interpretation given that myosin is only one of many proteins whose function is affected by thiol oxidation. For instance, the thiol oxidation of actin can disrupt the organisation of the actin filaments, leading to reductions in the force generating ability of the sarcomere (Milzani et al., 2000). The function of troponin is also altered when oxidised (Putkey et al., 1993). Finally, the thiol oxidation of tropomyosin affects the ATPase activity of actomyosin (Williams & Swenson, 1982). Although further work is required to corroborate this interpretation, these findings highlight further the importance of investigating the response of the thiol oxidation of individual proteins in addition to that of total proteins.

It is important to note that one potentially confounding factor with the muscle model adopted here is that the mean total protein thiol oxidation of resting muscles prior to contraction was higher than those measured in muscles which were immediately frozen after being removed from mice. This could represent a limitation with performing studies using in vitro muscle preparations given the evidence that the thiol oxidation of muscle proteins affects muscle contractile function (Chapters 2 and 3). Since the muscle preparations used here were already under increased oxidative stress compared to those
in intact animals, and considering the improvement in contractile force associated with DTT treatment, it is highly likely that muscle contractile performances measured in muscles from non-diabetic mice and diabetic mice were suboptimal.

In conclusion, this study shows that the maximal specific force of the EDL preparation is not reduced in Ins2Akita diabetic mice compared to non-diabetic mice despite higher thiol oxidation level of muscle proteins in diabetic mice. These findings suggest that the higher thiol oxidation level of proteins such as myosin in diabetic mice exposed to fatiguing stimulation might explain their lesser resistance to fatigue. However, given the limitations with using in vitro muscle preparations in this study, any attempt to extrapolate these findings to T1DM in humans or Ins2Akita diabetic mice should be performed with caution.
CHAPTER 5:

GENERAL DISCUSSION
5.1 GENERAL DISCUSSION

In recent years, there has been mounting evidence suggesting that reactive oxygen species (ROS) play some role in the development of skeletal muscle fatigue. However, the intracellular mechanism(s) by which ROS contribute to muscle fatigue is still unclear. One mechanism whereby ROS might play a role in muscle fatigue involves the reversible thiol oxidation of muscle proteins. Another mechanism implicates ROS-induced protein carbonylation which has recently been shown to be reversible in some situations (Matsunaga et al., 2008; Wong et al., 2008, 2010). Although the functions of several muscle proteins have been reported to be affected by these processes, it still remains to be determined whether the thiol oxidation levels of these and other proteins increase with fatiguing muscle stimulation. For this reason, the primary objective of Chapter 2 was to use the extensor digitorum longus (EDL) muscle preparation from rats to establish whether protein thiol oxidation level increases with muscle fatigue and to determine the extent to which this contributes to muscle fatigue. Given the importance of controlling for muscle damage and the possibility that short-lived transient rise in protein carbonylation might also contribute to fatigue, the second objective of Chapter 2 was to examine the response of protein carbonylation to fatiguing muscle stimulation and to evaluate the contribution of this process to fatigue.

The findings in Chapter 2 provide compelling evidence that reversible protein thiol oxidation rather than protein carbonylation is one of the mechanisms responsible for muscle fatigue under the experimental conditions adopted in this thesis. Firstly, total
protein thiol oxidation level increases in response to fatiguing stimulation, but not following sustained non-fatiguing stimulation. Secondly, unlike protein carbonylation responses, the recovery of muscle contractile performance after fatiguing stimulation is accompanied by a return of total protein thiol oxidation to pre-stimulation levels. Thirdly, both muscle fatigue and total protein thiol oxidation are more pronounced when fatiguing stimulation takes place in the presence of the thiol oxidising agent, diamide. Fourthly, muscle contraction performed with the thiol reducing agent, DTT decreases the magnitude of both muscle fatigue and total protein thiol oxidation. Among the many muscle proteins likely to be targeted by reversible protein thiol oxidation, the responses of individual proteins separated by gel electrophoresis suggest that the thiol oxidation of the muscle contractile proteins, myosin and actin might contribute to muscle fatigue.

Despite the rise in protein carbonylation levels with muscle fatigue, the results of Chapter 2 show that protein carbonylation and thus oxidative protein damage is unlikely to contribute to muscle fatigue under the experimental conditions adopted in this study. Firstly, muscle contraction in the presence of the thiol reducing agent or thiol thiol oxidising agent decreased and increased muscle fatigue without affecting protein carbonylation level. Secondly, the recovery of contractile following fatiguing stimulation is not associated with any fall in protein carbonylation level. It is important to stress, however, that these results do not necessarily imply that the fall in muscle contractile performance during exercise cannot involve an increase in oxidative protein damage as the extent to which proteins were damaged in this study might have been too low to affect muscle contractile performance. This is supported by the data on skinned muscle
fibers showing that a marked rise in protein carbonylation level can impair muscle contractile performance.

Although the reversible rise in the thiol oxidation level of muscle proteins contributes to muscle fatigue, it is estimated that only 20% of the overall loss of contractile force during fatiguing stimulation is from the contribution of protein thiol oxidation. This is suggested by the observation that the normalisation of the thiol oxidation level of muscle proteins with the antioxidant, DTT during fatiguing stimulation improves muscle contractile force by only 20%. This finding thus indicates that other factors play a more important role in muscle fatigue. One limitation, however, with these results is that they are based on experiments performed at 25 °C on isolated EDL muscles from rats. Since this temperature is markedly different from that of working muscles, and considering that the importance of other mechanisms of muscle fatigue (e.g. acidosis) have been shown to be temperature sensitive (Adams et al., 1991; Pate et al., 1995; Westerblad et al., 1997), this raises the question of the importance of the thiol oxidation of muscle proteins as a contributor of fatigue at physiological muscle temperature. For this reason, the objective of Chapter 3 was to determine whether protein thiol oxidation plays an important role in muscle fatigue at 34 °C due to greater ROS production and whether the exposure of protein thiol reducing agent, DTT during fatiguing stimulation at 34 °C affects protein thiol oxidation and muscle fatigue.

The results of Chapter 3 show that the thiol oxidation level of muscle proteins has a large effect on muscle contractility, thus suggesting that this mechanism has the potential to
play an important role in muscle fatigue at physiological temperature. Firstly, this is supported by the observation that total protein thiol oxidation level increases in response to fatiguing stimulation. Secondly, acute exposure to the thiol reducing agent, DTT improves muscle contractile performance with time while decreasing the thiol oxidation level of total muscle proteins including that of myosin, actin, troponin and tropomyosin. These results differ, however, from those in Chapter 2 in that muscle contractility can increase by as much as 87% in the presence of DTT, thus suggesting that reversible protein thiol oxidation has the potential to be a major contributor to muscle fatigue at physiological temperature. Unfortunately, the importance of this mechanism in contributing to fatigue could not be ascertained in this study. This is because the observation that total protein thiol oxidation levels prior to contraction in resting muscles were far above those found in vivo and together with the progressive temporal increase in muscle contractile force exposed to DTT during stimulation suggests that DTT is beneficial, in part, because it normalises muscle function rather than only opposing muscle fatigue.

The increase in the thiol oxidation of muscle proteins in response to fatiguing stimulation raises the obvious issue of the identity of the proteins involved. Close inspection of the responses of individual protein bands separated by gel electrophoresis suggests that the thiol oxidation of myosin might affect muscle contractile performance at physiological temperature, whereas the absence of changes in actin, troponin and tropomyosin suggests that these proteins are not involved. However, since DTT treatment and associated improvement in muscle contractile performance is accompanied by a fall in the thiol oxidation levels of myosin, actin, troponin, and tropomyosin, it is possible that the
thiol oxidation of actin, troponin and tropomyosin also play a role in muscle fatigue. These findings in mice at physiological temperature are thus consistent with those obtained in rats in Chapter 2.

The results of Chapters 2 and 3 that changes in the thiol oxidation level of muscle proteins can affect acutely muscle contractile performance raise the question of whether some of the medical conditions associated with impaired muscle function might also be linked with an increased in the thiol oxidation level of muscle proteins. Since there is compelling evidence for oxidative stress to be increased (Nishikawa et al., 2001; Evans et al., 2005; Kaneki et al., 2007) and muscle contractile performance to be impaired (Andersen et al., 1996; Andersen et al., 1997; Andreassen et al., 2006) in individuals with Type 1 diabetes mellitus (T1DM) as well as in animal models of diabetes (Chapter 4), the objectives of Chapter 4 were to determine whether the thiol oxidation level of muscle proteins is increased in the diabetic Ins2Akita mice model and to examine whether there is an association between muscle contractile performance and protein thiol oxidation level in this animal model.

Against expectations, there was no difference in maximal specific force between muscles from non-diabetic mice and diabetic mice despite the higher mean total protein thiol oxidation in muscles from diabetic mice. This might be taken as evidence that difference in the thiol oxidation level of muscle proteins are not necessarily associated with corresponding differences in muscle contractile performance. In particular, the observation that the thiol oxidation levels of actin and tropomyosin were higher in
muscles of diabetic mice suggests that the degree to which they were thiol oxidised was not high enough to affect muscle function. However, one important finding is that the thiol oxidation of myosin and troponin did not differ between muscles from non-diabetic mice and diabetic mice. Given the role played by myosin in muscle contraction, and previous findings that the oxidation of myosin inhibits myosin ATPase activity (Tiago et al., 2006), the absence of difference in the thiol oxidation of myosin between muscles from non-diabetic and diabetic mice might possibly explain their similar maximal specific force. Arguably, this interpretation holds as long as myosin responsiveness to thiol oxidation plays a more important role in determining contractile force. Although further work is required to determine whether this is the case, the findings with myosin and troponin highlight the importance of examining the response of individual proteins in addition to that of total proteins.

Despite the absence of difference in maximal specific force between the muscles of non-diabetic and diabetic mice, the latter was less resistant to fatigue. Interestingly, the finding that the total thiol oxidation level of muscle proteins attained after fatiguing stimulation in muscles from non-diabetic mice are comparable to that of muscles from diabetic mice prior to contraction could be taken as evidence that changes in protein thiol oxidation level may not play an important role in explaining the increased fatigue in diabetic mice. However, a different interpretation emerges when the thiol oxidation levels of individual proteins are compared between muscles from non-diabetic and diabetic mice. Although the thiol oxidation levels of actin, troponin and tropomyosin did not differ between fatigued muscles from non-diabetic mice and pre-fatigue muscles from diabetic
mice, the thiol oxidation of myosin is lower in pre-fatigue muscles from diabetic mice compared to fatigued muscles from non-diabetic mice. If the thiol oxidation of myosin were to play a major role in muscle force generation and fatigue, this observation alone would explain why resistance to muscle fatigue is lower in muscles from diabetic mice compared to muscles from non-diabetic mice despite comparable total protein thiol oxidation level. However, more work is required to corroborate this interpretation.

In conclusion, this thesis shows for the first time that the thiol oxidation level of muscle proteins increases in response to fatiguing muscle stimulation, and identifies experimental conditions where oxidative stress contributes to muscle fatigue through protein thiol oxidation rather than oxidative protein damage. This thesis also suggests that reversible protein thiol oxidation has the potential to be a major mechanism of fatigue at physiological temperature and that muscle fatigue is likely a consequence of the thiol oxidation of multiple proteins such as the contractile proteins. This might be part of a redox braking strategy, where protein thiol oxidation decreases contractile force in order to protect the cell against dangerous increases in ROS production. However, the findings of this thesis also highlight the limitations with using isolated muscle preparations as experimental models. In particular, there is a need for better models that normalise the protein thiol oxidation level of isolated muscle preparations at physiological temperature. Also, and more importantly, the findings described in this thesis raise the issue of investigating the physiological importance of the thiol oxidation of muscle proteins in muscle fatigue not only in healthy humans, but also in individuals afflicted by medical conditions known to impair muscle contractile function such as type 1 diabetes,
fibromyalgia, and chronic obstructive diseases to name a few. Finally, this thesis raises the future challenge of evaluating the processes that lead to the changes in the thiol status of muscle proteins and also the critical sulfhydryls in these proteins that affect their activities. In addition, the relative contributions of the different thiol oxidised proteins to muscle fatigue as well as the relative importance of thiol-mediated fatigue compared to the many other mechanisms of muscle fatigue remain to be investigated.
CHAPTER 6:

REFERENCES
5.1 REFERENCES


