The functional and molecular consequences of oxidation in the skeletal muscle myofilament

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November 2010
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THESIS DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Timothy Spencer and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Signed,

Timothy Spencer


I wish to thank my primary supervisor, Dr David Wilson. His mentorship and guidance during this time has been immeasurable and I will benefit from for the rest of my life.

I wish to thank Dr Giuseppe Posterino (La Trobe University, Victoria) for his supervision of Chapter 3 “Sequential effects of GSNO and H$_2$O$_2$ on the Ca$^{2+}$-sensitivity of the contractile apparatus of fast- and slow-twitch skeletal muscle fibers from the rat”.

I wish to thank Dr Louise Brown and James Cooke at the Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia. Recombinant proteins used in the current study were a gift from Louise Brown”s laboratory.

Finally I would like to thank my laboratory colleagues; Dr. Scott Copley, Kanchani Rajopadhyaya, Jessica Dunn, Ksenya Wojewidka, Yann Chan, Amenah Jaghoori and Joanne Eng, for creating such a friendly and productive laboratory environment.
THESIS ABSTRACT

It is becoming increasingly evident that redox state leading to post-translational modifications of structural proteins, enzymes and ion channels can cause activation or inhibition of cellular function (Andrade et al., 1998a, Jackson, 2008, Kelly et al., 1996). While low levels of nitric oxide (NO) synthesised by endothelial and neuronal nitric oxide synthase have been shown to provide a beneficial effect to tissues, the elevated release of NO accompanying inflammation has a detrimental effect, resulting in dysfunction (Khanna et al., 2005). We investigated the functional consequence and molecular substrate of NO and another potentially harmful reactive oxygen species, H$_2$O$_2$, on the skeletal muscle myofilament.

In a rat model we used functional myography of demembranated single fast- and slow-twitch skeletal muscle fibers to examined the consequence of the addition of the free radical NO and reactive oxygen species H$_2$O$_2$ on the Ca$^{2+}$ sensitivity of the myofilament. The reversibility of oxidative modifications following NO or H$_2$O$_2$ treatment was examined using the general anti-oxidant dithiothreitol. Isoelectric focusing combined with SDS-PAGE separation of proteins investigated the post-translational modification of free-radical exposed myofilament proteins. Molecular substitution of endogenous troponin C (TnC) with WT cardiac/slow TnC or C84S TnC, incapable of being oxidized at Cys84, investigated the molecular and functional consequence of oxidation of TnC at Cys84.
Exposure of fast-twitch muscle fibers to NO resulted in a decrease in Ca$^{2+}$ sensitivity, while H$_2$O$_2$ had the opposite effect, increasing Ca$^{2+}$ sensitivity. In contrast, slow-twitch fibers were insensitive to both NO and H$_2$O$_2$. Following myofilament exposure to NO (~2 μM) proteomic analysis revealed that many proteins underwent post-translational modification, including myosin light chain (LC$_{20}$) and TnC. Molecular substitution of endogenous fast-twitch TnC with WT-cardiac/slow TnC demonstrated a similar sensitivity to NO as WT skeletal muscle. In contrast TnC, non-oxidizable at Cys84, rendered fast-twitch skeletal muscle insensitive to NO.

Many myofilament proteins, including myosin light chains were identified as being post-translationally modified by NO exposure, however, molecular substitution experiments clearly identify TnC, specifically residue Cys84 as the functional substrate responsible for fast-twitch skeletal muscle sensitivity to NO. Although slow-twitch muscle contains the same isoform of TnC, it was insensitive to NO. This suggests that slow-twitch muscle may have a greater capacity for anti-oxidant defense than fast-twitch muscle. The contrasting increase in Ca$^{2+}$ sensitivity following H$_2$O$_2$ to the decline caused by NO demonstrates that not all oxidative molecules act alike, possibly targeting differing substrates and causing differing post-translational modifications.
<table>
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<tr>
<td>BH4</td>
<td>tetrahydrabiopterin</td>
</tr>
<tr>
<td>([\text{Ca}^{2+}]_{\text{cyt}})</td>
<td>cytoplasmic calcium concentration</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium-induced-calcium-release</td>
</tr>
<tr>
<td>DHPR</td>
<td>dihydropyridine receptor</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitroso-glutathione</td>
</tr>
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<td>H(_2)O(_2)</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
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<tr>
<td>IRI</td>
<td>ischemia-reperfusion injury</td>
</tr>
<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
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<tr>
<td>MI</td>
<td>myocardial infarct</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>O(_2)(^{-})</td>
<td>super oxide</td>
</tr>
<tr>
<td>OH(^{-})</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>ONOO</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>pCa</td>
<td>(-\log [\text{Ca}^{2+}])</td>
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<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
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<td>RyR</td>
<td>ryanodine receptors</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SNAP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>SOD</td>
<td>super oxide dismutase</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>TnC</td>
<td>troponin C</td>
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<td>TnI</td>
<td>troponin I</td>
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<td>TnT</td>
<td>troponin T</td>
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Literature Review
Introduction

Contraction of cardiac and skeletal muscle is a highly regulated process involving; the generation of ATP, regulation of extracellular and sarcoplasmic reticulum (SR) Ca\(^{2+}\) entry and Ca\(^{2+}\)-dependent conformational changes of structural, regulatory and motor proteins. It is becoming increasingly evident that redox state leading to post-translational modifications of structural proteins, enzymes and ion channels can cause activation or inhibition of cellular function (Andrade et al., 1998a, Jackson, 2008, Kelly et al., 1996). However, many cells are able to maintain their redox balance despite the generation of reactive oxygen (ROS) and reactive nitrogen species (RNS) primarily due to the production of endogenous anti-oxidants and reductants. Anti-oxidants convert oxidized molecules into harmless electron stable molecules or remove modifications caused by an oxidative reaction. For example superoxide dismutase (SOD) enzymatically converts the reactive oxygen species superoxide (O\(_2\)\(^{\cdot}\)) to the less reactive hydrogen peroxide (H\(_2\)O\(_2\)) and oxygen (McCord and Fridovich, 1969).

\[
O_2^{\cdot\cdot} + O_2^{\cdot\cdot} + 2H^+ \rightarrow O_2 + H_2O_2
\]

Catalase is a selective anti-oxidant enzyme involved in the catalytic conversion of H\(_2\)O\(_2\) to H\(_2\)O and oxygen (Murrant and Reid, 2001).

\[
H_2O_2 \rightarrow 2H_2O + O_2
\]

It therefore follows that molecular, cellular and systems function can be regulated by an imbalance of oxidant and/or reductant.

Following from the Nobel Prize winning work of Furchgott, Murad and Ignaro (Furchgott, 1999) it is well recognized that endothelial derived nitric oxide (NO) is an
important vasodilator which improves blood flow and consequently cardiac and skeletal muscle perfusion and function (Kelm and Schrader, 1990, Rees et al., 1989, Stamler and Meissner, 2001). In contrast, following myocardial infarction or skeletal muscle damage infiltrating neutrophils and mast cells activate their inducible nitric oxide synthase (NOS) to generate a very large release of NO (Khanna et al., 2005, Laroux et al., 2001).

In addition, several studies have demonstrated that application of exogenous NO donors to functional cardiac and skeletal muscle preparations have a negative influence on the contractile function. Using the mixed fiber-type diaphragm muscle Kobzik et al. identified that nitric oxide synthase inhibitors such as nitro-L-arginine improve contractile function including the force-frequency relationship, and that these increases in contractile function could be reversed by the exogenous NO donors S-nitroso-N-acetylcysteine (SNAC) and sodium nitroprusside (SNAP) (Kobzik et al., 1994b). Similarly, in electrically stimulated cardiomyocytes, the application of SNAP or superfusion with an NO containing physiological solution attenuated contraction (Brady et al., 1993). These data illustrate that high levels of NO can have a detrimental effect on one or more elements involved in excitation-contraction coupling in muscle.

In fast-twitch fibers, the contractile apparatus is sensitive to ROS including H$_2$O$_2$. Exogenous application of H$_2$O$_2$ has been shown to enhance the contractile function of myocytes, increasing the Ca$^{2+}$-sensitivity of the contractile apparatus likely through oxidation of sulfhydryl groups (Andrade et al., 1998a, Lamb and Stephenson, 1994).
It is important to recognise that, as well as species specific differences, fast- and slow-twitch skeletal muscles are functionally different in several respects for example: fast-twitch muscle have a higher peak amplitude of Ca\(^{2+}\) release (fast, 18.5±0.5 vs. slow, 6.4±1.0 µM Δ[Ca\(^{2+}\)] ) and a shorter time between ½ maximum contraction and ½ maximum relaxation (fast, 4.9±0.3 vs. slow, 7.7±0.6 ms) (Baylor and Hollingworth, 2003). Herein, all studies were conducted with rat fast- and slow-twitch skeletal muscle. Fast-twitch fibers are more sensitive to fatigue, whereas slow-twitch fibers show a fatigue resistance that appears to be due in part to a lack of sensitivity to various metabolites involved in fatigue in fast-twitch fibers. This key feature enables one to functionally discriminate between the fast- and slow-twitch fiber types. To date, there has been no systematic comparison of the effects of both ROS and NO on the Ca\(^{2+}\)-sensitivity of both fast- and slow-twitch fiber types, moreover there is very little known about the role of NO in slow-twitch fiber Ca\(^{2+}\)-sensitivity.

While several studies have shown the capacity of proteins involved in excitation-contraction coupling to be oxidised, and documented an association of contractile dysfunction with elevated ROS/RNS, very few studies have demonstrated a direct link between the observed contractile dysfunction and oxidation of specific proteins. In this study we identify the functional effects of both NO, through the use of exogenous donors, and the ROS H\(_2\)O\(_2\), on fast- and slow-twitch skeletal muscle myofilament function. Importantly we have extended the study to include
identification of the functional substrate that causes the NO-mediated decline in myofibril Ca\textsuperscript{2+}-sensitivity in fast twitch skeletal muscle.
Oxidative balance

Free radical production and oxidation

Free radicals are chemical species possessing an unpaired electron. They are formed in a variety of ways including: 1) homolytic cleavage of a covalent bond; 2) the loss of a single electron; 3) the addition of a single electron. Electron transfer is the more common process, as homolytic cleavage requires high-energy input, such as high temperature or UV light.

Free radicals or broadly, oxidants cause either the addition of a charged molecule like oxygen or the loss of electrons from a substrate. The consequences of which include changes in protein conformation and protein-protein interactions altering cellular function. Similar post-translational modifications, such as phosphorylation (the addition of a phosphoryl group, PO\(_4\)), are important signaling mechanisms, however, unlike phosphorylation many oxidative reactions are not catalyzed by an enzyme and may not have evolved with the same degree of target specificity (Mannick and Schonhoff, 2002).
Reactive oxygen species

As a consequence of basal muscle metabolism oxidants are always being generated, in fact Davies et al. (1982) have provided evidence of lipid peroxidation and oxidant production during rest, which become elevated following submaximal, exhaustive activity independent from inflammation. Although free radical production was known to occur well before 1970, the diversity of specific oxidant species produced within muscle were not identified till almost 20 years later. Superoxide and hydrogen peroxide were found to be released in contracting diaphragm muscle, as identified by the use of the selective anti-oxidant enzymes SOD and catalase, reducing 2”,7”-dichlorofluorescein, an intracellular fluorochrome probe for oxidation (Reid et al., 1992). The hydroxyl radical was also detected in contracting skeletal muscle (O’Neill et al., 1996) and nitric oxide generation has been identified in skeletal muscle (Balon and Nadler, 1994).

Molecular sources of reactive oxygen species in skeletal muscle are varied and depend on the physiological state of the muscle. Glycolytic metabolism results in the production of ATP from glucose and ADP but, as a by-product of the conversion of glyceraldehyde 6-phosphate to 1,3-bisphosphoglycerate, also produces two unstable hydrogen cations which can go on to form $\text{H}_2\text{O}_2$. Significantly, interruption of the electron transport chain, resulting in incomplete reduction of oxygen to $\text{H}_2\text{O}$, also produces oxidants in the form of the superoxide anion (Boveris et al., 1972, Loschen et al., 1971). Superoxide is generated at complex I and III of the electron transport chain by electron transport intermediates such as ubiquisemiquinone (Nishikawa et al., 2000). Changes in the mitochondrial environment such as increased $[\text{Ca}^{2+}]$ following
ischemia or a high potential difference in the proton gradient which drives ATP synthase, can disrupt electron transport and actuate \( \text{O}_2^- \) generation (Korshunov et al., 1997, Turrens, 1997). It was widely reported that 2-5% of total oxygen consumed by mitochondria result in the generation superoxide (Boveris and Chance, 1973, Loschen et al., 1974), more recent reports suggest this value may be lower (St-Pierre et al., 2002) but nevertheless still present. As striated muscles have a high density of mitochondria, oxidants are largely produced during cell metabolism.

A number of other sources of \( \text{O}_2^- \) generation have been identified (Fig A.1). Superoxide is also generated by a cardiac and skeletal muscle sarcoplasmic reticulum (Cherednichenko et al., 2004, Xia et al., 2003)) and plasma membrane (Javesghani et al., 2002) associated enzyme, NAD(P)H oxidase. Reid and colleagues have described a phospholipase \( \text{A}_2 \)-dependent superoxide generation distinct from the calcium-dependent phospholipase \( \text{A}_2 \) ROS production also identified in skeletal muscle (Gong et al., 2006, Nethery et al., 1999). Xanthine oxidase is also a source of \( \text{O}_2^- \) generation, however, its specific role in physiological or pathophysiological oxidative stress remains equivocal.

Superoxide is highly unstable, with a half-life (\( t_{1/2} \)) of 1 \( \mu \)sec (Reth, 2002) before reacting with either \( \text{H}_2\text{O} \) to form \( \text{H}_2\text{O}_2 \) or with \( \text{H}_2\text{O}_2 \) form the hydroxyl anion (Fig A.1). Peroxide is membrane permeant and more stable with a relatively long \( t_{1/2} \) of 1 msec (Reth, 2002). However, in a pure solution it reaches a stable equilibrium due to the limited available molecules in the buffer to be oxidized. Although \( \text{H}_2\text{O}_2 \) is cytotoxic it is considered a relatively weak oxidizing agent, nevertheless, it readily
generates other very toxic free radicals such as the hydroxyl radicals (OH\(^-\)) particularly in the presence of catalytic transition metals iron (Fe\(^{2+}\)) commonly released during ischemia (Pattwell and Jackson, 2004).

**Figure A.1. Free radical sources within the muscle and associated cells.** Nitric oxide is released by nitric oxide synthase (NOS) enzymes such as endothelial NOS (eNOS) and inducible NOS (iNOS). Superoxide (O\(_2^•\)) has multiple cellular sources including; NADPH oxidase, xanthine oxidase, phospholipase A2 and mitochondria. The uncoupling of eNOS through limitation of substrate or co-factors such as tetrahydrabioppterin (BH4) leads to O\(_2^•\) production. Superoxide and NO react to form peroxynitrite (ONOO). The endogenous anti-oxidant, super oxide dismutase (SOD) catalyses the conversion of O\(_2^•\) to \(\text{H}_2\text{O}_2\) which may go on to form OH\(^-\) or in the presence of catalase break down to \(\text{H}_2\text{O}\) and \(\text{O}_2\).
The relative potency of reactive oxygen species (Halliwell, 1987).

$\text{OH}^-$ (highly reactive) $> \text{O}_2^*$ (selectively reactive) $> \text{H}_2\text{O}_2$ (weak non-radical)
**Reactive nitrogen species**

As every cell in the body contains mitochondria, superoxide production is ubiquitous. Similarly the free radical, nitric oxide (NO), is produced in most organs in the body. Nitric oxide is synthesized by the enzyme, nitric oxide synthase (NOS) from the substrates L-arginine and oxygen, utilizing several co-factors including NADPH and tetrahydrobiopterin (BH4), to form L-citrilline and NO (Fig A.1). There are three known isoforms of nitric oxide synthase, two constitutively expressed Ca$^{2+}$/CaM dependent isoforms, endothelial-NOS and neuronal-NOS and the Ca$^{2+}$ independent, inducible-NOS. Endothelial-NOS mediated NO is produced in every organ of the body simply because every organ contains blood vessels and endothelial cells. Similarly most organs in the body are innervated and consequently produce NO through a neuronal NOS-dependent mechanism. However, under normal physiology this NO is used as a direct vasodilator or as a neurotransmitter.

Evidence of the expression of multiple isoforms of NOS has also been found in skeletal muscle. Staining with a monoclonal antibody for nNOS, identified its presence in the sarcolemma membrane, while staining for eNOS displayed co-localization with mitochondrial markers suggesting eNOS expression in mitochondrial membranes (Kobzik et al., 1994b, Kobzik et al., 1995). Along with endothelial and neuronal cells, many tissues including muscle contain another potential source of NO synthesized from resident neutrophils expressing iNOS, activated via cytokines liberated during the inflammatory response.
Nitric oxide is continuously generated in skeletal muscle, with production increasing during contraction (Balon and Nadler, 1994) likely due to increased mitochondrial activity, neuronal activation, or endothelial release as one increases vasodilatation during exercise. Nitric oxide is a membrane permeable free radical and readily reacts with other molecules and proteins as it attempts to reach a ground state. Importantly, in the presence of superoxide, nitric oxide will preferentially react to form peroxynitrite (Fig A.1) a potent vasoconstrictor and highly reactive and damaging free radical, (Halliwell et al., 1999).

\[ \text{O}_2^\bullet + \text{NO} \rightarrow \text{ONOO}^- \]
Maintaining the balance: anti-oxidants

Redox state

Anti-oxidants function to delay, prevent or reverse the oxidation of cellular substrates by oxidants. The balance of anti-oxidants and oxidants in a cellular system determines the redox state, or level of oxidative post-translational modification present within the cell. The redox state of a cellular system is linked to many pathological states particularly when genes encoding anti-oxidant enzymes have been mutated. The pathological disorder is manifest in a variety of systems and includes; idiopathic cardiomyopathy, neurological disorders and cancer. Particularly relevant in our aging population is the finding that diabetics are reported to have decreased levels of protective anti-oxidants and an increase in oxidative stress (Borcea et al., 1999, Maritim et al., 2003).

Levels of anti-oxidants can also vary with age, diet and exercise. Anti-oxidant production is up regulated in response to prolonged muscle conditioning and down regulated with de-conditioning (Clanton et al., 1999). Dietary intervention has also been studied and used to alter anti-oxidant levels within the body; however, the benefits of dietary interventions remain equivocal (Bjelakovic et al., 2004). This may be complicated by the administration of anti-oxidants, which normally act synergistically with endogenous anti-oxidants and that alone, have the potential to act as oxidants themselves once initially oxidized (Herbert, 1994).
Endogenous anti-oxidants

A number of anti-oxidants act on specific ROS and are compartmentalized throughout the cytoplasm and within organelles such as the mitochondria, enabling them to act near the source of the specific ROS production or important target proteins. The major anti-oxidants in the body include catalase, superoxide dismutase, glutathione and peroxidase. Several isoforms of superoxide dismutase are found within the cell and in the extracellular space as well as within the mitochondrial matrix (Weisiger and Fridovich, 1973a, Weisiger and Fridovich, 1973b). Superoxide dismutase works specifically on the superoxide radical to form the less reactive oxidant, hydrogen peroxide (Sen, 1995). Hydrogen peroxide can be further reduced by the hydrogen peroxide dismutase, catalase, to O\textsubscript{2} and H\textsubscript{2}O (Deisseroth and Dounce, 1970). Glutathione is a less specific anti-oxidant, spontaneously scavenging a wide variety of ROS. It is synthesized within the body from the amino acids L-cysteine, L-glutamic acid and glycine. Glutathione itself has a free cysteine moiety that donates an electron to a substrate, with the consequence of reducing the substrate. During this reaction glutathione becomes an unstable, oxidative form, but it readily reacts with other reactive glutathione molecules to form stable glutathione disulfide. Glutathione reductase and NADPH are responsible for regenerating glutathione. Glutathione acts in concert with other anti-oxidants, reducing them from their radical form allowing them to continue at act as anti-oxidants (Sen, 1995).

Other anti-oxidants function to remove the post-translational modifications caused by some oxidants. Protein disulfide reductase, glutaredoxin and thioredoxin reductase remove intramolecular and mixed di-sulfide bonds caused by oxidation.
Exogenous anti-oxidants

Exogenous anti-oxidants form a second line of defense against oxidative changes and are primarily obtained as nutrients or supplements in the diet. Exogenous anti-oxidants have become the focus of a large number of clinical studies as evidenced in a variety of published meta-analysis (Bjelakovic et al., 2004, Miller et al., 2005, Vivekananthan et al., 2003). However, many exogenous anti-oxidants are consumed as part of normal dietary intake. Increasing the intake of exogenous anti-oxidants such as omega-3 and 6 fatty acids, vitamins C, D and E and lutein have been suggested to increase anti-oxidant levels within the body, with a potential beneficial effect (Heinonen and Albanes, 1994, Landrum et al., 1997, Morris et al., 1998, Simopoulos, 1991, Zandi et al., 2004). However, meta-analysis of exogenous anti-oxidant clinical trials provides evidence that the efficacy of such treatments remains equivocal (Bjelakovic et al., 2004, Miller et al., 2005, Vivekananthan et al., 2003).
**Oxidation as a cellular signal**

Similar to enzyme mediated phosphorylation, spontaneous oxidation can cause post-translational modifications of proteins and lipids leading to conformational changes and alterations in protein or lipid function. For example NO is a cellular signal often associated with vascular control. Nitric oxide produced from Ca\(^{2+}\)-dependent eNOS in the endothelium is a potent vasodilator. Nitric oxide reacts with transition metals such as heme iron or iron-sulfur centers to form metal-NO products. Specifically this NO-dependent reaction is used to increase the activity of enzymes including guanylate cyclase leading to increased cyclic guanosine monophosphate production (cGMP) (Furchgott, 1999). The signal propagated by activating cGMP-dependent ion channels, protein kinases and phosphodiesterases mediates among other functions, a reduction in intracellular [Ca\(^{2+}\)] leading to smooth muscle relaxation and increase in organ blood flow. On the other-hand, peroxynitrite derived from NO has the opposite effect. Specifically, the uncoupling of eNOS through oxidation or the lack of substrates or co-factors such as BH4, as seen with aging and sedentary lifestyle leads to the production of superoxide rather than NO (Stroes et al., 1998). Superoxide reacts with the residual NO present to produce peroxynitrite. This limits the bioavailability of NO reducing vasodilatation, amplified by the fact that peroxynitrite is also a vasoconstrictor.
The role of ROS in cellular signaling

Reactive oxygen species and the resulting oxidation can promote the generation of an anti-oxidant defense mechanism which results in the up regulation of anti-oxidants in mitochondria. Specifically oxidation has been shown to stimulate mitochondrial biogenesis via activation of transcription factors for genes encoding glutathione S-transferase, glutathione peroxidase and glutathione reductase, all involved in glutathione biosynthesis and cycling (Motohashi and Yamamoto, 2004, Piantadosi and Suliman, 2006). Reactive oxygen species are also thought to regulate SOD and catalase activity. In fact, muscle contraction increases ROS production, while post-exercise is associated with increase in catalase and SOD activity in both mouse (McArdle et al., 2001) and human muscle (Khassaf et al., 2001). The increased activity of catalase and SOD may represent an increase in the amount of enzyme present. Interestingly prior supplementation with Vitamin C (an anti-oxidant) reduces these responses (Khassaf et al., 2001), suggesting the level of oxidation and ROS is directly involved in increasing the amount of catalase and SOD present following activity.
The role of RNS in cellular signaling

Nitric oxide, or more specifically NO derived species, readily react with protein thiols, forming a S-NO bond or nitrosylation. The formation of nitrosothiols is favoured in cysteine thiols, however, not all free-thiol cysteines become nitrosylated (Lane et al., 2001, Martínez-Ruiz and Lamas, 2004). A level of specificity is seen in protein nitrosylation, enabling it to be involved in cellular signaling. Cysteines with an “acid-base motif” either in the primary sequence (Stamler et al., 1997) or in proximity in the tertiary structure provide a more favorable reaction (Ascenzi et al., 2000), providing specificity through a consensus motif, e.g. Hist-Cys96-Asp forms the NO binding site of the β-subunit of haemoglobin (Hess et al., 2001). As expected the consequences of nitrosylation are also concentration dependent and the co-localization of reactive proteins with eNOS and nNOS provides some specificity in signaling, however, it appears that specificity may be lost in iNOS mediated reactions due to NO generation being several orders of magnitude greater than eNOS or nNOS.

Nitrosylation has been identified as a potential mediator in a number of signaling pathways. Nuclear magnetic resonance of P21^{RAS}, an activator of NF-κB, has identified a nitrosylated cysteine located in a nucleotide-binding motif. Although nitrosylation did not apparently alter the protein structure the enzyme activity is increased during the process of nitrosylation (Williams et al., 2003). Nitrosylation has also been implicated in modification of the p50 subunit of NF-κB inhibiting DNA binding (delaTorre et al., 1999, Marshall et al., 2004, Matthews et al., 1996), activation of hypoxia-inducible factors (Sumbayev, 2003, Yasinska and Sumbayev,
2003) and the inhibition of the ryanodine receptor (Mészáros et al., 1996, Zahradníková et al., 1997).
Pathophysiological roles and sources of oxidants

Oxidants are produced under a number of pathophysiological conditions including heat stress, enzyme uncoupling and inflammation resulting from conditions including sepsis, ischemia-reperfusion injury, trauma or activity induced muscle injury. Under these conditions the production of oxidizing agents is in excess of the ability of endogenous reductants to quench them and consequently results in the cellular ROS and RNS to interact with a number of cellular proteins causing post-translational modifications that may cause cellular activation or dysfunction (De Belder et al., 1993, Li and Shah, 2004, Paulus et al., 1994).

Proteomic assays of blood serum have shown proteins of the troponin complex to be modified. Analysis of troponin T and troponin I released from the myocardium, have identified both as being post-translationally modified (Labugger et al., 2000, McDonough et al., 1999, McDonough et al., 2001), indicating the proteins of the contractile myofilament may be modified during ischemia reperfusion injury and other pathologies of excess NO and ROS production.
Inflammation and neutrophil activation

Inflammation and sepsis are both associated with an increase in ROS and RNS (Callahan et al., 2001, Vane et al., 1994). Both pathologies activate invading neutrophils that lead to the activation of iNOS and consequent NO generation and substantial amounts of ROS. Muscle injury, in particular crush injury triggers a similar local inflammatory response, activating invading neutrophils and macrophages. The release of ROS and NO at the site of muscle crush injury is associated with preparation of the tissue to allow for regeneration (Malech and Gallin, 1987). In the inflammatory response triggered by sepsis and IRI, the increased NO and ROS are considered a means to destroy any invading pathogens and damaged cells, however, the magnitude of the ROS release is very often overwhelming resulting in collateral damage to previously healthy tissue (Bone, 1991).

Muscle dysfunction during sepsis

Nitric oxide and ROS production during sepsis is associated with muscle dysfunction (Callahan et al., 2001, Fujimura et al., 2000, Nethery et al., 1999, Supinski et al., 1993). Cecal ligation and perforation, or endotoxin induced sepsis have been shown to decrease both the maximum force produced and Ca\(^{2+}\) sensitivity of muscle strips and single muscle fibers isolated from the diaphragm (Callahan et al., 2001, Fujimura et al., 2000, Nethery et al., 1999), EDL and soleus (Supinski et al., 1993). The addition of NOS inhibitors, such as L-NAME and free-radical scavengers, such as polyethylene glycol-superoxide dismutase (PEG-SOD) and apocynin prevents muscle dysfunction.
suggesting that oxidant production is associated with muscle dysfunction (Callahan et al., 2001, Shindoh et al., 1992, Supinski et al., 1993).
Ischemia reperfusion injury

The physiological response to local ischemia as a result of coronary artery disease, myocardial infarction, claudication or during heavy exercise in skeletal muscle is for the vascular endothelium to release the potent vasodilator, NO. However, with prolonged ischemia, tissue damage occurs leading to an inflammatory response and subsequent release of large amounts of NO and ROS from inflammatory cells (Jordan et al., 1999). This damage is further exacerbated during reperfusion as invading neutrophils enter the area accompanying the restored blood flow. The larger proportion of cellular damage during IRI is associated with the NO and ROS production during reperfusion (Khanna et al., 2005).

Limited substrate supply or oxidation of enzymes can also lead to the production of free radicals through the uncoupling of enzymes, including uncoupling of NOS. In addition when the co-factor BH4 becomes limited as often occurs in aging, (Sindler et al., 2009) NOS produces the superoxide free radical rather than NO (Heinzel et al., 1992). Production of the superoxide anion by NOS further decreases the bioavailability of NO due to the preferential reaction of NO with superoxide forming peroxynitrite (Beckman and Koppenol, 1996).

Importantly, the interaction of NO and superoxide to form peroxynitrite or NO with heme proteins to form heme protein adducts is more favorable than the interaction of superoxide with superoxide-dismutase (SOD) leading to the production of H₂O and O₂. The formation of peroxynitrite leads to the nitration of proteins and the reduced
bioavailability of NO, and reduces its ability to function as a physiological vasodilator and signaling molecule. If substrate supply is restricted due to vascular constriction or ischemia, the reduced bioavailability of NO, will further compound the ischemic condition, as the peroxynitrite formed functions as a potent vasoconstrictor.

**Muscle dysfunction during ischemia-reperfusion injury**

Ischemia-reperfusion injury (IRI) occurs when blood flow is restored to a previously ischemic tissue. Although necessary to salvage ischemic tissue, the restoration of blood flow actually causes further, possibly greater, damage than the ischemic event as a result of the inflammatory response to reperfusion (Khanna et al., 2005). Nitric oxide, although cytoprotective during ischemia, is also responsible for the damaging inflammatory response in IRI. As with sepsis and other inflammatory responses, iNOS is activated and NO is produced in excess (Khanna et al., 2005). Nitric oxide production has been shown to peak following 15 minutes of ischemia, persisting for 2-3 hours in skeletal muscle within a mouse hind limb IRI model (Brovkovych et al., 1999). Nitric oxide production has been shown to surge again beginning two hours into reperfusion as iNOS from invading neutrophils and macrophages is activated (Barker et al., 2001, Messina et al., 2000). Nitric oxide inhibitors, when administered during the last 5 minutes of ischemia and the first 2 hours of reperfusion have beneficial effects on contractile function and post-ischemic blood flow (Ikebe et al., 2002). The beneficial effects of NOS inhibition have also been confirmed through administration at time periods just prior to reperfusion and up to 3 hours post-reperfusion (Zhang et al., 1997).
Fundamental mechanisms regulating striated muscle contraction and relaxation

Cross-bridge cycling

Cross-bridge cycling describes the repeated process by which the myosin molecular motor causes sliding of the thin filament, leading to contraction. As the understanding of interactions of myosin, actin and other associated regulatory proteins has improved so has the model of cross-bridge cycling evolved. Early studies using light microscopy, observed changes in the size of I-band and H-zone during contraction and produced a model of two states; 1) steric hindrance by ATP, prevented myosin from binding to actin and prevented contraction, 2) ATP hydrolysis by the myosin ATPase and release of the hydrolyzed phosphate imparted a conformational change which enabled actin and myosin to bind forming the cross-bridge (Huxley and Hanson, 1943).

The two-state model evolved into the three state model of: blocked, closed and open (McKillop and Geeves, 1993). Biochemical and structural studies suggested that, in the absence of Ca\(^{2+}\) tropomyosin prevented actin-myosin binding, the „blocked” state (McKillop and Geeves, 1993). Upon Ca\(^{2+}\) binding troponin C on the filament moves into the „closed” state (McKillop and Geeves, 1993) in which TnI detaches from actin (Tao et al., 1990), shifting tropomyosin by ~30° and myosin is able to bind actin through a weak electrostatic interaction. With myosin binding weakly to actin, the filament moves into the „open” state (McKillop and Geeves, 1993). Myosin binding
weakly to actin shifts the tropomyosin a further $10^\circ$ allowing for a strong bond formation and a conformational change in the myosin head, the power stroke, shifting the actin filament $\sim 4 \text{nm}$ or more (Molloy et al., 1995), accompanied by the release of Pi (Gordon et al., 2000).

Hydrolysis of ATP by the myosin-ATPase generates the energy required to drive the cross-bridge cycling and contraction. The movement of myosin along the actin filament is a multi-step process; 1) myosin ATPase binds ATP, 2) the myosin head dissociates from actin 3) ATP is hydrolyzed forming myosin-ADP-Pi, 4) the myosin head associates with actin in a weakly bound form, 5) in a $\text{Ca}^{2+}$-troponin dependent manner, the weak association isomerizes to a strong bond, 6) in the strongly bound configuration myosin-ATPase produces a rotation in the myosin head and neck region (Cooke and Holmes, 1986), generating movement, a force of $\sim 1.7 \text{pN}$ under isometric conditions (Molloy et al., 1995) and releases Pi, 7) myosin-ATPase, bound to actin, releases ADP returning to the initial state (Gordon et al., 2000).
Figure A.2. Cross-bridge cycling. 1) Myosin ATPase binds ATP, 2) the myosin head dissociates from actin 3) ATP is hydrolyzed forming myosin-ADP-Pi, and in a Ca$^{2+}$-troponin dependent manner binds to the actin-myosin binding site, 4) myosin-ATPase produces a rotation in the myosin head and neck region and releases Pi, 6) myosin-ATPase, bound to actin, releases ADP returning to the initial state. Modified from a web based animation http://www.unmc.edu/physiology/Mann/mann14.html (08/02/2011)
The role of ion channels and Ca$^{2+}$

Although not the focus of the following studies it is important to recognize the Ca$^{2+}$ entry pathways essential to Ca$^{2+}$-mediated regulation of contraction. Neuronal signals originating from the central nervous system or in the case of cardiac muscle, from the sinoatrial node are transmitted to the motor neurons. At the motor neuron the signals are chemically transmitted across neuromuscular junction creating action potentials that then propagate along the muscle cell surface membrane and t-tubule system. Action potentials trigger a conformation change in surface membrane L-type Ca$^{2+}$ channels. The conformational change, either directly or via a Ca$^{2+}$ intermediate, transmit the signal to the intracellular Ca$^{2+}$ store, which then releases free Ca$^{2+}$ into the cytosol resulting in contraction. Striated muscle cells contract uniformly due to the rapid conductance of an action potential and subsequent influx of Ca$^{2+}$ along the sarcolemma and the t-tubules, which extend internally in both transverse and longitudinal orientations. (Franzini-Armstrong and Porter, 1964, Posterino et al., 2000).

High resolution electron microscopy and deconvolution confocal microscopy has confirmed that the t-tubules form tight junctions with the terminal cisternae of the sarcoplasmic reticulum (SR), a large Ca$^{2+}$ store within the myocyte which envelopes the myofilament (Block et al., 1988). At the tight junctions of the t-tubular membrane, voltage sensitive L-type Ca$^{2+}$ channels, also known as dihydropyridine receptors (DHPR) become activated by membrane depolarization. In turn activation of L-type Ca$^{2+}$ channels on the t-tubule membrane activate the Ca$^{2+}$ channels of the SR, known as Ryanodine receptors (RyR) due to their sensitivity to this alkaloid. The
precise mechanism by which the L-type Ca\textsuperscript{2+} channel activates the RyR-mediated Ca\textsuperscript{2+} release from the SR is not fully understood in skeletal muscle and differs somewhat from cardiac muscle due to differing expression of RyR isoforms (Berridge et al., 2000, Niggli, 1999).

In cardiac muscle the plasma membrane L-type Ca\textsuperscript{2+} channels are activated and allow for extracellular Ca\textsuperscript{2+} entry. Extracellular Ca\textsuperscript{2+} binds to and activates the RyR2 in a process known as Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR) (Fabiato, 1983). It is theorized that as local concentrations of Ca\textsuperscript{2+} increase in the vicinity of the RyR Ca\textsuperscript{2+} competes with and displaces an inhibitory Mg\textsuperscript{2+} bound to the RyR causing channel opening and SR Ca\textsuperscript{2+} release into the cytosol (Lamb and Stephenson, 1992, Laver et al., 1997). The release of SR Ca\textsuperscript{2+} further induces RyR2 opening and further Ca\textsuperscript{2+} release. The close proximity of the L-type Ca\textsuperscript{2+} channels and the RyR limits diffusion distance and allows for rapid signal transduction (Block et al., 1988, Carl et al., 1995, Franzini-Armstrong et al., 1999).

In skeletal muscle no or exceedingly low levels of extracellular Ca\textsuperscript{2+} is required to initially activate the RyR1, although CICR is thought to aid in the propagation and amplification of the signal (Jacquemond et al., 1991, Stern et al., 1997). The L-type Ca\textsuperscript{2+} channels and RyR of the SR are embedded in the membranes in a patterned array with direct contact either between the L-type Ca\textsuperscript{2+} channel and the RyR or via intermediate linking proteins including the FK506 proteins (Block et al., 1988, Protasi et al., 1998). Similar to the cardiac RyR2, skeletal RyR1 opening is inhibited
by Mg\(^{2+}\) binding (Lamb and Stephenson, 1992). Activation of the L-type Ca\(^{2+}\) channel removes this inhibition, possibly through a conformational change.

Exclusive of cross sectional area, the strength of a skeletal muscle cell”s contraction is largely governed by the quantity of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{\text{cyt}}\)) released from the SR. The free Ca\(^{2+}\) concentration inside the SR is as high as \(\sim1\text{mM}\) or more and additional Ca\(^{2+}\) is sequestered and stored by the Ca\(^{2+}\) binding proteins calsequestrin and calreticulin (Berridge et al., 2003) and is released upon activation of the RyR. Activation of the SR bound Ca\(^{2+}\)-ATPase (SERCA2) enables reuptake of [Ca\(^{2+}\)\(_{\text{cyt}}\)) into the SR at which point free SR Ca\(^{2+}\) becomes bound to calsequestrin anchored to scaffolding proteins including triadin and junctin, near the RyR at the terminal cisternae (Berridge et al., 2003). This compartmentalization of Ca\(^{2+}\) allows for a high concentration of Ca\(^{2+}\) to be localized to the Ca\(^{2+}\) release channels away from the site of Ca\(^{2+}\) sequestration. Single cell Ca\(^{2+}\) fluorescence, site directed mutagenesis and gene knock-down studies have shown calsequestrin and associated proteins, coupled to the RyR also act as an internal Ca\(^{2+}\) sensor, modifying Ca\(^{2+}\) release based on the SR Ca\(^{2+}\) content (Kalyanasundaram et al., Koizumi et al., 1999, Oddoux et al., 2009). Following repolarization of the T-tubules and inactivation of DHPR and RyR Ca\(^{2+}\) release channels, the SERCA2 pumps activity transports free Ca\(^{2+}\) back into the SR, ceasing the contraction and relaxing the muscle (Melzer et al., 1995).
As described above, skeletal muscle contraction is a complex function involving Ca$^{2+}$ signaling via L-type Ca$^{2+}$ channels, activation of RyR and conformational changes in myofilament proteins. Changes in the function of the proteins and channels involved regulate contraction.

At the same time, historical and recent evidence has demonstrated that oxidants have the ability to modify skeletal muscle channels and myofilament proteins. These modifications can either positively or negatively regulate muscle function.

Proteomic techniques have identified that a variety of the myofilament proteins become modified when in an oxidising environment (Dalla Libera et al., 2005, Vescovo et al., 2000). Exposure to a NO donor such as SNAP and NEM has been shown to reduce ATP hydrolysis by the myosin ATPase (Perkins et al., 1997), however, the functional consequence to force production was not investigated. Spin labelled myosin ATPase in a rabbit psoas myofilament, showed that when over 75% of the SH-1 sites are modified a significant change in contractile function was observed (Crowder and Cooke, 1984). In addition the light chains reconstituted with myosin, following 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) exposure, form intramolecular disulfide bonds (Wolff-Long et al., 1993) however, again no functional impact was investigated. Actin in its globular form has several cysteine residues that can be oxidised, however, when polymerised into functional actin filament the detection of oxidation is reduced or abolished (Liu et al., 1990). These data illustrate while many studies have shown the capacity of myofilament proteins to be oxidised, few experiments directly link contractile dysfunction with oxidation of a specific myofilament protein.
To simplify our investigation of the myofilament basis for oxidant mediated muscle dysfunction, we chose to use a demembranated myofibril, isolating the contractile apparatus from the Ca$^{2+}$ signaling and metabolic activity of the muscle. Combined with proteomic and molecular substitution techniques we are able to directly link post-translational modification of a specific myofilament protein with a decline in skeletal muscle myofilament function.
The Contractile Apparatus

Variation in muscle types

Skeletal and cardiac muscle share the similar Ca\(^{2+}\)-dependent activation of contraction, however, isotype specific differences in the expression of contractile protein isoforms leads to alterations in the kinetic properties of the distinct muscle isotypes. Consequently, while cardiac muscle is easily identified as tissue specific, protein isoform expression provides a mechanism for characterizing skeletal muscle types.

Historically several different approaches have been used to classify muscle fiber type including; myosin ATPase rate, metabolic substrate use and oxidative potential (Essen et al., 1975, Peter et al., 1972). Skeletal muscle fibers, broadly classed as either fast- or slow-twitch muscle, are further delineated into subgroups that can be identified by metabolic activity. Type 1, slow-twitch fibers utilize oxidative phosphorylation while fast twitch fibers can be divided into two classes: 1) type 2A, oxidative-glycolytic, and 2) type 2B, glycolytic. Interestingly, the metabolic pathway used reflects the muscle fibers susceptibility to fatigue following prolonged used. Type I, slow-twitch muscle are fatigue resistant, type 2A, fast-twitch are also fatigue resistant, while type 2B, fast-twitch are fatigue-sensitive (Armstrong, 1988).
The myofilament

While Ca\(^{2+}\) entry into the cytosol is the primary signaling mechanism activating cardiac and skeletal muscle contraction, the molecular machinery that drives the contraction of a muscle cell are the myofilaments. Cardiac and skeletal muscle myofilaments span the muscle cell longitudinally and are a structured into a complex of motor and regulatory proteins arranged as bidirectional filaments. The thick filament consists predominantly of the motor protein, myosin, while the thin filament consists of the Ca\(^{2+}\)-sensitive regulatory troponin protein complex, positioned on a backbone of filamentous actin.

Myofilaments are arranged into sarcomere units, visible under light microscopy (Fig A.3). The ends of the sarcomere, Z-lines, attach neighbouring sarcomeres and anchor the thin filaments. Myosin thick filaments, are arranged in parallel, and make up the A band, the ends of which overlap the thin filament, where cross-bridge cycling occurs. The I-band and H-zone describe areas in which the thin and thick filaments do not overlap, respectively. During contraction these zones are compressed as the Z-lines, attached to the thin filaments, are pulled towards the centre of the sarcomere while the thin filament slides over the myosin thick filament (Huxley and Niedergerke, 1954).
Figure A.3. The myofilament sarcomere unit. Z-lines, attach neighboring sarcomeres and anchor the thin filaments. Myosin thick filaments, running in parallel, make up the A band, the ends of which overlap the thin filament. The I-band and H-zone describe areas in which the thin and thick filaments do not overlap, respectively. Modified from (Sherwood, 2009).
Myosin

Myosins consist of a family of 15 or more classes of motor proteins (Hodge and Cope, 2000). The thick filament of both cardiac and skeletal muscle is comprised of myosin II. In muscle, the thick filament of the myofilament is composed of a bipolar polymer containing several hundred myosin proteins and associated myosin binding proteins including the myosin light chain (LC\textsubscript{20})(Gordon et al., 2000).

Myosin II is made up of three functional subdomains: (1) the motor domain or S1 site, which interacts with actin and hydrolyses ATP, (2) the flexible neck domain, which binds light chains or calmodulin and is involved in the power stroke and (3) the tail domain, which serves to anchor and positions the motor domain.

The thick filament is a bipolar polymer, in which the myosin proteins lie in parallel, half orientated in one direction and the other half in the opposite direction such that the myosin tail subdomains line up end-to-end in the centre (Fig A.5). Each myosin molecule itself is made up of two identical subunits. The subunits consist of the three functional subdomains, the tail subdomains entwine, pairing the subunits, with the motor domain heads protruding toward the actin-binding site (Fig A.4).

The molecular machinery of the myosin head drives the movement of actin along the myosin filament. Myosin ATPase-mediated hydrolysis of ATP to ADP and Pi provides the free energy that powers the conformational change in the myosin head known as the power stroke (Molloy et al., 1995). During the power stroke myosin, bound to the actin thin filament, slides the thin filament towards the tail of the myosin
protein. As the thick filaments are arranged in a bipolar orientation, the myosin heads at either end slide the thin filaments closer together, causing the cell to shorten and contract (Fig A.5).
Figure A.4. Myosin II motor-protein. Each myosin molecule itself is made up of two identical subunits. Myosin II is made up of three functional subdomains: (1) the motor domain or S1 site, which contains the actin-binding site and myosin ATPase, (2) the flexible neck domain, which binds light chains or calmodulin and is involved in the power stroke and (3) the tail domain, which anchors the motor domain.
Figure A.5. Actin movement during contraction. During the power stroke myosin, bound to the actin thin filament slides the thin filament towards the tail of the myosin protein. As the thick filaments are arranged in a bipolar orientation, the myosin heads at either end slide the thin filaments closer together.
The Ca$^{2+}$ dependent binding of myosin to actin and the subsequent conformational change mediated by ATP hydrolysis is a rate limiting step and important site of regulation of contraction. Muscle myosins are regulated in a number of ways depending on isoform expressed and the tissue in which it is found. Within cardiac and skeletal muscle, actomyosin binding and myosin ATPase activity is regulated by the troponin-tropomyosin complex of proteins associated with the thin filament and myosin motor domain, binding site on actin. In the absence of Ca$^{2+}$ the troponin-tropomyosin complex prevents actin-myosin binding, necessary for ATP turn-over at the S1 site and contraction. As intracellular Ca$^{2+}$ increases it binds to the TNC complex causing a conformational change which permits acto-myosin interaction and cross-bridge cycling.

Myosin associated proteins can also alter myosin function. In smooth muscle it is well recognized that phosphorylation of the light chains of myosin by myosin light chain kinase causes contraction (Kamm and Stull, 1985, Kureishi et al., 1997). In contrast, phosphorylation of the myosin light chains of skeletal muscle, by addition of calmodulin (2 $\mu$M and 6 $\mu$M) and myosin light chain kinase (0.15 $\mu$M and 0.5 $\mu$M respectively), in both rabbit and rat skinned, skeletal muscle fibers has been shown to increase the sensitivity to Ca$^{2+}$ and submaximal isometric force generation, although not cause contraction alone (Metzger et al., 1989, Sweeney and Stull, 1990). More recent studies have speculated that the single cystein on myosin LC$_{20}$ could be a target of oxidation and contribute to dysfunction.
The major isoforms of myosin heavy chain (MHC) found in skeletal muscle are: MHCIβ in slow-twitch, and MHCIIa, MHCIIId and MHCIIb in the three subgroups of fast-twitch fibers. Although generally grouped into distinct classes, muscle fibers can co-express two or more major MHC isoforms, forming a continuum between fiber types (Schiaffino et al., 1989, Billeter et al., 2005).

Other contractile myofilament proteins are expressed to match the myosin heavy chain isoform. Myosin light chains are expressed as six different isoforms in skeletal muscle: MLC1f, MLC2f and MLCf3 in fast-twitch muscle and MLC1sa, MLC1sb and MLC2s in slow-twitch muscle (Pette and Staron, 2000, Schiaffino et al., 1989, Billeter et al., 2005). Troponin T and troponin I isoform expression also varies based predominantly on the MHC isoform present (Bottinelli et al., 1991).
Table A.1. Fiber type, myosin and myosin associated protein isoform expression.

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Myosin Heavy Chain</th>
<th>Myosin Light Chain</th>
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<tbody>
<tr>
<td>Slow</td>
<td>MHC1β</td>
<td>MLC2s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MLC1sa</td>
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<tr>
<td></td>
<td></td>
<td>MLC1sb</td>
</tr>
<tr>
<td>Fast</td>
<td>MHCIIa</td>
<td>MLC1f</td>
</tr>
<tr>
<td></td>
<td>MHCIIId</td>
<td>MLC2f</td>
</tr>
<tr>
<td></td>
<td>MHCIIb</td>
<td>MLC3f</td>
</tr>
</tbody>
</table>
Isoform specific differences in myosin expression affect the rate of ATP hydrolyses. For example, human skinned muscle fibers classified as type I or slow (type I MHC isoform), type II A (type II A MHC) and type II B (type IIB MHC) from biopsies of the rectus abdominus and vastus lateralis, had ATPase activity (mmol l$^{-1}$ s$^{-1}$) ranging from 0.62±0.08 (I MHC), 1.16±0.13 (II A MHC), to 2.46±0.35 (II B MHC) at 35°C (Bottinelli et al., 1994, Stienen et al., 1996).
Actin

Actin is a common cytoskeletal and scaffolding protein found in all eukaryotic cells and provides a path along which the many different forms of myosin motor proteins travel. The actin in striated muscle is made up of globular actin (G-actin) molecules that form a polymer of filamentous actin (F-actin). Actin contributes to a range of cellular processes including cell motility, division and signaling, as well as providing structure and shape to the cell (Doherty and McMahon, 2008).

The polymerization of G-actin into F-actin is a complex and tightly regulated process. At the core of polymerization are four steps: 1) the activation of G-actin through the binding of ATP, 2) the formation of an actin nucleus consisting of a dimer or trimer of ATP-bound G-actin, 3) bidirectional elongation of the actin nucleus as activated G-actin monomers bind at both the barbed and pointed ends of the actin nucleus, and 4) the annealing of and coiling of paired, elongated filaments (Pollard and Cooper, 1986).

Associated with the actin filament and an important regulator of actin polymerization are capping proteins, such as gelsolin, villin and severin. The binding of capping proteins to ends of the forming actin filament have been show to regulate the elongation of the filament, inhibiting further actin binding, but also preventing the reserve or loss of G-actin from the filament (Pollard and Cooper, 1986). Within striated muscle, capping of the actin filament occurs at the Z-line where apposing
actin filaments meet. These barbed ends are capped by CapZ and are linked by α-actinin, forming the Z-line (Littlefield and Fowler, 1998).

Actin makes up a major constituent of the muscle myofilament. The thin filament of striated muscle is made up of two parallel filamentous F-actin polymers arranged in a tight alpha helical pattern (Fig A.6a). Associated with the actin filaments are several actin-bound, regulatory proteins including tropomyosin and the troponin complex that regulate actin-myosin binding and thus contraction in a Ca\(^{2+}\)-dependent manner.
Tropomyosin

Tropomyosin is primarily found in association with actin filaments. Over 40 isoforms exist and tropomyosin is widely distributed in the cytoskeletal network among cell types but has a prominent role in the contractile myofilament of smooth, cardiac and skeletal muscle. There are at least four isoforms found in muscle, two in striated muscle and two in smooth muscle (Lees-Miller and Helfman, 1991).

Tropomyosin exists as either a homo- or heterodimer depending on the muscle type (Lehrer, 1975, Yamaguchi et al., 1974). Two alpha-helical chains are arranged as a coiled-coil (Yamaguchi et al., 1974), with the long tropomyosin chains overlapping in a head to tail fashion as a continuous polymer along the thin filament in a close association with F-actin. (Fig A.5b)

Tropomyosin lies in the long pitch groove of F-actin, partially obstructing the myosin binding sites (Moore et al., 1970). Electron microscopy and three-dimensional image reconstruction has identified that, through steric hindrance, tropomyosin regulates the strength of actin-myosin binding and hence contraction in cardiac and skeletal muscle (Vibert et al., 1997).

The position of tropomyosin along the actin filament can be regulated through post-translational modifications or associated proteins. Partially phosphorylated tropomyosin has been found in both cardiac and skeletal, rat and rabbit, muscle homogenates (Heeley et al., 1982). Phosphorylation causes a greater avidity for head-
tail polymerization in a purified protein preparation and promotes myosin-ATPase activity to a greater extent, in a reconstituted actin and myosin S1 fragment ATPase assay (Heeley et al., 1989). In skeletal and cardiac muscle, tropomyosin binds to the F-actin associated, Ca$^{2+}$-sensitive troponin complex. Upon Ca$^{2+}$ binding, a conformational change in the troponin complex alters the binding affinity of tropomyosin to troponin, shifting tropomyosin, allowing strong actin-myosin binding and contraction (Vibert et al., 1997, Xu et al., 1999).
Figure A.6. The arrangement of actin and tropomyosin polymers. a) Globular actin polymerizes to form the actin filament, which consists of two parallel filamentous F-actin arranged in a helical pattern. The thin filament of striated muscle is made up of two parallel F-actin polymers. Associated with the actin filament are regulatory proteins the inhibit actin-myosin binding in a Ca\(^{2+}\)-dependent manner. b) The continuous tropomyosin polymer, overlapping in a head to tail fashion sits in the long-pitch groove of F-actin. Tropomyosin partially obstructs actin-myosin binding in a Ca\(^{2+}\)-troponin dependent fashion.
The Troponin Complex

The troponin complex is found in both skeletal and cardiac muscle in association with the thin filament. The troponin complex binds to F-actin and tropomyosin at regular intervals with a stoichiometry of one troponin complex to one tropomyosin. The troponin complex is made up of three subunits; 1) troponin I (TnI), 2) troponin T (TnT) and 3) troponin C (TnC) (Greaser and Gergely, 1973). Calcium-binding to TnC causes a series of conformational changes relayed through the troponin complex that ultimately alter protein binding-affinities. Although not fully understood, the process subsequently displaces tropomyosin from the myosin-binding site of actin, which enables actin-myosin binding and muscle contraction (Tao et al., 1990, Tobacman, 1996, Vibert et al., 1997, Xu et al., 1999).
Troponin I

Troponin I binds to actin and the other troponin subunits, TnT and TnC. Reconstituted myofilament experiments have shown TnI functions to inhibit the myosin ATPase in a Ca\(^{2+}\)-independent manner, preventing the detachment of the myosin motor head from the actin-myosin binding site (Potter et al., 1995). Myofilament consisting of the actomyosin S-1 fragment and Tm exhibit a basal level of ATPase activity, this activity is abolished when TnI is included (Potter et al., 1995). The addition of TnC reverses the TnI inhibition of ATPase activity but does not raise ATPase activity above basal levels, even in the presence of Ca\(^{2+}\) (Potter et al., 1995). This was initially believed to be the mechanism of regulating contraction.

Troponin I functions as an anchor for the troponin-tropomyosin-actin complex. In the presence of Ca\(^{2+}\) the binding of TnC to TnI is strengthened, reportedly increasing between 15-100 fold in skeletal muscle (Tobacman, 1996), while the TnI-actin interaction weakens, and fluorescence resonance energy transfer and photo cross-linking experiments indicate the distance between actin and TnI increases (Tao et al., 1990). The altered binding affinity and movement of TnI causes the movement of the tropomyosin-troponin complex away from the actin-myosin binding site, allowing for actin-myosin binding and contraction (Fig A.6).

Cellular damage to cardiac or skeletal muscle results in TnI leaking from the cell into the extracellular space and eventually the blood. Currently blood TnI is used as a clinical diagnostic for cellular damage following myocardial infarct and crush injuries.
**Troponin T**

Troponin T is the largest of the troponin subunits and binds to actin, tropomyosin and the troponin complex. The COOH-terminus of TnT binds to TnC, TnI and tropomyosin (Perry, 1998). The NH2-terminus sits at the region where the tropomyosin tail and preceding tropomyosin head overlap (Gordon et al., 2000). In the presence of Ca\(^{2+}\) the binding of the COOH-terminus of TnT and TnC is strengthened, while weakening the binding to Tm (Gordon et al., 2000).

Although TnT has a structural role it also appears to potentiate acto-myosin ATPase activity in the presence of Ca\(^{2+}\). Reconstitution experiments including Tm, TnI and TnC and actomyosin-S1 ATPase assays in the presence of Ca\(^{2+}\) show only a basal level of ATPase activity (Potter et al., 1995). However, the inclusion of TnT, completing the troponin complex, potentiates acto-myosin ATPase activity in a Ca\(^{2+}\) dependent manner to a maximal increase of ~170% over basal activity (Potter et al., 1995). The increased activity may be facilitated by either a Ca\(^{2+}\)-dependent interaction between TnT and TnC or a Ca\(^{2+}\) dependent change in the interaction between TnC and TnI, transmitted through TnT (Potter et al., 1995).
Troponin C

The troponin C subunit is the Ca\(^{2+}\) sensor protein of the troponin complex. The binding of Ca\(^{2+}\) to low affinity binding sites of TnC initiates a cascade of conformational changes that lead to actin-myosin binding and contraction. Two isoforms of TnC are found in skeletal and cardiac muscle. All fast-twitch fibers express skeletal TnC (skTnC) while slow-twitch fibers and cardiac muscle both express the cardiac isoform (cTnC) (Wilkinson, 1980). Unlike other contractile proteins, co-expression of both isoforms of TnC in one tissue has not been observed.

The distinct TnC isoform expression difference in slow- and fast-twitch muscle is utilized in identifying fiber type. Muscle fibers contract in response to the divalent cation, Strontium (Sr\(^{2+}\)), however, slow-twitch fibers on average have a sevenfold greater sensitivity to Sr\(^{2+}\) (O'Connell et al., 2004). SDS-PAGE analyses of single fibers and reconstitution experiments have demonstrated that this increase in sensitivity is associated with the presences of cardiac/slow-twitch TnC (Morimoto and Ohtsuki, 1987, O'Connell et al., 2004, Yamamoto, 1983, Hoar et al., 1988).

Both isoforms of TnC are EF hand proteins with a typical dumbbell shape having two EF hands linked by a flexible linker region and are both Ca\(^{2+}\)-sensitive proteins required for the initiation of contraction (Fig A.7). In fact, molecular substitution experiments i.e., the exchange of skeletal TnC with cardiac TnC, in a skeletal muscle model, indicates that the TnC isoforms function similarly (Moss et al., 1986a, Moss et al., 1991).
The COOH-terminus of TnC is anchored to the NH2-terminus of TnI independent of [Ca\(^{2+}\)]. In contrast, the NH2-terminus of TnC binds to the inhibitory and COOH-terminus of TnI in a Ca\(^{2+}\)-dependent manner (Farah and Reinach, 1995).

The COOH-terminal contains two high affinity structural Ca\(^{2+}\) binding sites, while the NH2-terminal EF hand contains the regulatory, low affinity binding site(s). In skeletal muscle the two high affinity Ca\(^{2+}\) binding sites have an affinity of \(~2.0 \times 10^7\) M\(^{-1}\) (Potter and Gergely, 1975), while in cardiac TnC the affinity is \(3 \times 10^8\) M\(^{-1}\) (Holroyde et al., 1980).

Cardiac/slow TnC contains a single low affinity binding-site, while fast skeletal TnC contains two low-affinity binding sites (Holroyde et al., 1980, Potter and Gergely, 1975) which cause the conformational change leading to contraction. The large Ca\(^{2+}\) mediated conformational change in the NH2-terminus exposes an extensive number of hydrophobic residues or a „sticky” patch, which interacts with the inhibitory and COOH-terminal regions of TnI (Gagne et al., 1995).

Interestingly, when substituted into a fast-twitch skeletal muscle fiber, purified cardiac TnC infers a higher Ca\(^{2+}\)-sensitivity in the contractile apparatus than skeletal TnC, with the original sensitivity restored when the cardiac TnC was subsequently removed and skeletal TnC re-introduced (Moss et al., 1986a, Moss et al., 1986b).
Small differences in species-specific homologs of TnC regulate Ca$^{2+}$-sensitivity. For example, the Ca$^{2+}$-sensitivity of the contractile apparatus of trout cardiac muscle is >10 fold greater than that of a mammalian heart at the same temperature (Churcott et al., 1994), in part due to a two-fold increase in Ca$^{2+}$-affinity of trout TnC. This difference comes about from a five amino acid difference in the NH2-terminal domain (Gillis et al., 2007), none of which actually reside within the low-affinity binding domain but likely still functions to cause conformational changes in protein-protein interactions. It is also possible that post-translational modifications will alter the affinity of TnC regulatory, low affinity Ca$^{2+}$-binding sites and Ca$^{2+}$-sensitivity of the contractile apparatus.
Figure A.7. Troponin C ribbon structure. Cardiac/slow-skeletal (left - 1AJ4 (Sia et al., 1997)) and fast-twitch skeletal muscle (right - 1TN4 (Houdusse et al., 1997)) troponin C in the Ca$^{2+}$ bound structure. Both isoforms of TnC are EF hand proteins with a typical dumbbell shape having two EF hands linked by a flexible linker region. Cysteine sites are highlighted in grey.
As skeletal muscle contraction is the result of a complex signaling pathway involving many potential points of regulation, the following studies utilize a simplified, isolated single fiber model. This simplified model allows for the analysis of the functional consequence of ROS/RNS on the contractile proteins without considering the complexity of regulatory actions due to plasma membrane/SR of Ca\textsuperscript{2+} signaling or metabolic activity.

Oxidative stress includes modification of a variety of redox sensitive molecules, discussed above. The reactive nature and oxidative modification of the oxidative molecules varies and as such we utilize two very distinct oxidants, H\textsubscript{2}O\textsubscript{2} and NO.

Although functionally similar, slow- and fast-twitch muscle are known to have distinctly different responses to stimuli that cause fatigue and some forms of oxidative stress. We investigate the effects of exogenous oxidants on both fast- and slow-twitch muscle types with an aim to identify different susceptibility to NO-mediated oxidative dysfunction.

We move beyond the basic functional experiments and utilize molecular substitution and proteomic techniques to identify the functional substrate responsible for the NO-mediated decline in Ca\textsuperscript{2+}-sensitivity in fast-twitch muscle fibers.

This technique not only identified the functional, myofilament associated substrate but also allows for speculation on the basis for the differences in slow- and fast-twitch fiber responses to oxidative stress. The finding that the substitution of the slow-twitch isoform of the functional substrate into a fast-twitch muscle fiber does not abolish the NO-mediated decline in Ca\textsuperscript{2+}-sensitivity suggests that a greater abundance of anti-oxidant enzyme(s) or molecules may be associated with slow-twitch muscle fibers.
General Methods
Single muscle fiber myography

Premise

Demembranated single muscle fiber myography allows for the measurement of contractile forces independent from metabolism, regulation of $\text{Ca}^{2+}$ entry pathways, central nervous system and the influence of cytosolic proteins. This is in stark contrast to the complexity of whole muscle perfusion techniques which are useful in providing information on the response of an entire muscle organ including neural influence, muscle blood flow and cellular metabolites. In the presence of exogenously applied ATP, demembranated single muscle fiber myography produces accurate, reproducible measurements of isometric contractile force in response to an external $\text{Ca}^{2+}$ stimulus. A significant advantage of the demembranated single fiber myography is the near microscopic size of the fiber (50μm) that allows for rapid diffusion of exogenous substances throughout the sample.

Muscle Dissection and Mounting

All experiments were performed according to the guidelines and with the approval of the University of Adelaide Animal Ethics committee. Male, Hooded Wistar rats; 4-6 months old or 300-350g were euthanized in a CO$_2$ chamber. Forceps, surgical scissors and a scalpel were used to remove hind limb muscles overlaying the extensor digitorum longus (EDL) and soleus. Following exposure of the EDL or soleus the connecting tendons were cut and the muscle removed, carefully to grip only the
tendons. Isolated muscles were immediately pinned in a petri dish at slight tension (<95% resting tension) under paraffin oil (Chem-supply, Australia) and kept on ice. Paraffin oil prevents the muscle dehydrating.

Under a light microscope single fibers were isolated by making an incision into the muscle belly. Fine surgical forceps were used to gently separate bundles of multiple fibers away from the main muscle body. Subsequently, single fibers were then isolated from these smaller muscle bundles. Using fine forceps the sarcolemmal membrane was removed, 10-0 suture silk (Deknatel), was tied to the free end. The single muscle fiber was cut free from the muscle bundle and a second suture tied to the remaining free end. The single fibers were kept under slight tension (<5% stretch) before being mounted on the force transducer.

Individual fibers were mounted on an isometric force transducer by placing the surgical sutures around either end of a stationary pin and force transducer. The resting length of the fiber was measured using a graticule mounted on to the microscope and the fiber stretched to 120% resting length before the diameter of the muscle fiber was measured as previously documented (Lamb and Stephenson, 1994, Dutka et al., 2005, Endo, 1972). Once attached the fiber was immersed in a Perspex™ bath containing a K-EGTA (in mM: K⁺, 126; Na⁺, 36; EGTA⁻², 50; total ATP, 8; creatine phosphate (CP), 10; total Mg²⁺, 10.3; HEPES, 90; pH 7.10; pCa (-log₁₀ [Ca²⁺]), <9.0) solution containing 2% Triton-X 100 for 10 minutes to solubilize the remaining membranous compartments. The fiber was then washed free of cytosol and remnant membranes
and organelles in fresh K-EGTA buffer for 2 minutes to remove the residual Triton-X 100 prior to the commencement of experiments.
Figure B.1. A single muscle fiber dissected from the muscle body. The muscle body is pinned at slight tension and under paraffin oil. A single fiber is isolated from the muscle body and suture silk tied to either end before mounting into a force-transducer.
Apparatus

The muscle fiber myograph consisted of a force transducer (AME801, 2kHz resonance frequency, SensoNor Horten, Norway) with an adjustable slide. Using suture silk single fibers were attached to a stationary pin, on the transducer with a second suture used to connect the free end of the fiber to the adjustable slide which enabled one to adjust the resting length of the fiber (Fig B.2).

Beneath the force transducer was a height adjustable platform. A series of Perspex™ 2 ml baths on a linear slide enabled one to rapidly change the muscle bathing solution.
Figure B.2. A single muscle fiber mounted on a force-transducer. The single muscle fiber is mounted between a stationary pin and a pin connected to a force-transducer on an adjustable slide. The adjustable slide allows for adjustment of the muscle length.
**Ca\(^{2+}\) activation and contractile function**

The force-Ca\(^{2+}\) relationship was determined by activation of the fiber in a series of fixed Ca\(^{2+}\) concentration solutions buffered with EGTA. These covered a pCa range of pCa 7.0 to pCa 4.5. Fibers were initially incubated in nominally zero Ca\(^{2+}\), K-EGTA solution and prior to treatment the Ca\(^{2+}\) chelating EGTA was removed by briefly washing in a K-HDTA solution (in mM: K\(^+\), 126; Na\(^+\), 36; HDTA\(^2-\), 50; total ATP, 8; creatine phosphate (CP), 10; total Mg\(^2+\), 8.5, EGTA, 1; HEPES, 90; pH 7.10; pCa (-log\(_{10}\) [Ca\(^{2+}\)], <9.0). HDTA is a non Ca\(^{2+}\) chelating analog of EGTA. The fiber was then sequentially immersed in a series of solutions of increasing [Ca\(^{2+}\)], the fiber was moved to the next higher [Ca\(^{2+}\)] once tension reached a plateau. Following peak force was achieved the fiber was completely relaxed in a K-ETGA solution for at least 1 minute prior to repeating the activation sequence. The activation sequences were highly reproducible with only a small reduction in maximum Ca\(^{2+}\) activated force occurring with successive stimulation (~2% per sequence). The activation sequence was repeated at least twice per treatment.
**Experimental treatments**

*Sr\(^{2+}\) mediated identification of fast- vs slow-twitch fibers*

The soleus muscle contains predominantly slow-twitch fibers, however, there are a small number of fast-twitch fibers. To verify the fiber type isolated from both EDL and soleus muscle, single fibers were exposed to a Sr\(^{2+}\)-EGTA solution (pSr 5.4). Fast-twitch fibers produce little or no force in Sr\(^{2+}\)-EGTA solution while slow-twitch muscle fibers produce near maximal force (Bortolotto et al., 2000, O'Connell et al., 2004).

*Nitric oxide donor treatment*

The nitric oxide donor GSNO was made fresh each hour as a 10 mM stock in K-HDTA and further diluted to 1 mM. Fibers were exposed to a 1 mM GSNO solution for 2 minutes, followed by a washout period of 2 minutes in K-EGTA solution.

The amount of NO liberated from 1 mM GSNO was measured in K-HDTA solution at 23 °C using an Apollo 4000 NO analyzer system (World Precision Instruments) with a NO sensor. The peak NO concentration liberated immediately upon dissolving 1 mM GSNO in the K-HDTA solution was 14 μM with a half life of approximately 10 minutes. Following 20 minutes, NO production was such that concentration was 1-4 μM and stable for a further 40 minutes. SNAP (2 mM; an alternate NO donor) liberated a peak [NO] of 2.1 μM after 25 minutes and then gradually decayed with a
half-life of 30 minutes. Experiments were conducted during the period where the NO concentration was between 1-4 μM.

**Hydrogen peroxide treatment**

Hydrogen peroxide was diluted to a 1 M stock (in water) from a 3 M aqueous solution and then diluted to 10 mM in 2 ml of K-HDTA solution. Fibers were exposed to H₂O₂ whilst being simultaneously contracted in a pCa 6.0 solution to enhance the Ca²⁺-sensitivity effects of H₂O₂ (Posterino et al., 2003). Hydrogen peroxide was added to the K-EGTA, K-HDTA and pCa 6.0 solutions. Following from Posterino et.al (2003), the fiber was activated and relaxed repeatedly for 5 minutes, resulting in approximately 2 minutes of exposure during contraction.

**Troponin C extraction and replacement**

Troponin C was extracted and replaced using a method modified from Moss et al. 1985. Following an initial Ca²⁺-activation control sequence the fiber was relaxed in K-EGTA solution for 2 minutes. Partial extraction was completed by bathing the fiber in a 1 ml low ionic strength solution containing Tris (20 mM), EDTA (5 mM) and DTT (1mM) for 10 minutes. Following washout with K-HDTA buffer the force-Ca²⁺ relationship was re-determined as described above. Recombinant TnC was combined into the fiber by bathing in K-EGTA solution containing ~1 mg/mL recombinant TnC and 25mM DTT for 1 hour. This was followed by a 2 minutes wash in K-EGTA solution to remove excess TnC.
Data analysis

The data for each activation sequence within a fiber was individually analysed. Sub-maximal responses were normalized to the maximum Ca\(^{2+}\)-activated force in each sequence. The data obtained from each pair of activation sequences was then averaged and plotted using GraphPad Prism v4.01 (GraphPad Software, San Diego, CA). The force-pCa data was fitted with a modified Hill equation with parameters of maximum and minimum set to 100% and 0% respectively. From the resulting curve the pCa\(_{50}\) (the pCa at which 50% maximum force is produced) along with the pCa\(_{20}\) and pCa\(_{80}\), and the Hill Coefficient were determined. The effect of a treatment was assessed by measuring the change in pCa\(_{20}\), pCa\(_{50}\) and pCa\(_{80}\) relative to the values before treatment in the same fiber (Spencer and Posterino, 2009).

Similarly, in recombinant TnC substitution experiments the forces generated by fibers were normalized to the maximum force produced in the initial Ca\(^{2+}\) activation sequence prior to TnC extraction. Measuring the pCa\(_{50}\) of treated fibers in comparison to untreated fibers enabled assessment of the treatment effects.

The results are presented as means ± SEM for a sample of \(n\) fibers. Statistical significance was examined using either a student’s unpaired t-test or repeated measures one-way analysis of variance where appropriate with a significance level of \(P<0.05\).
Proteomic techniques and analysis

Separation of proteins using SDS-PAGE

Premise

SDS-PAGE is the separation of proteins according to their size within a polyacrylamide gel matrix. In the presence of the ionic detergent, sodium dodecyl sulfate (SDS), proteins become coated in the detergent anions and become largely negatively charged. When an electric field is applied to protein samples in the polyacrylamide matrix, the negative charge flows from the negative cathode in the upper buffer chamber, through the gel into the lower buffer chamber, attached to the positive anode. Since SDS imparts a near equal negative charge to all proteins, this technique causes globular proteins to be fractionated based on size with smaller proteins migrating more rapidly.

Polyacrylamide gel composition and preparation

Polyacrylamide gels were prepared by polymerizing monomeric acrylamide with N,N-methylene-bis-acrylamide (bis-acrylamide), as the cross-linker. The polymerization is initiated by the addition of a free radical generator ammonium persulfate (APS) and catalyzed by N,N,N,N-tetramethylethylenediamine (TEMED). Separation of proteins is largely dependent on the size of pores formed in cross-linked
polyacrylamide. Gels are usually referred to as total acrylamide as a percentage (w/v). In general, pore size decreases with increasing % total acrylamide. In general 7.5% polyacrylamide gels provide reasonable separation of proteins 300 - 100 kilodaltons (kDa), with 15% matrix being suitable for proteins less than 100 kDa, individual porosity can be further tailored to meet the needs of the experiment by changes to the percentage acrylamide or acrylamide relative to bis-acrylamide cross-linker.

Gels were cast 0.75 – 1.5 mm in thickness between glass plates. Gels were either a 15% acrylamide composition or a gradient gel (outlined below). Gradient gels consist of a 20% acrylamide solution slowly mixed with a 7.5% acrylamide solution with the aid of a peristaltic pump that provides for a more linear separation of proteins.

Briefly and referring to Figure B.4, Perspex™ chambers connected in series were filled with 17 and 16ml of 7.5% (a) and 20% (b) polyacrylamide solutions. Solutions mix in chamber (b), reducing the % polyacrylamide present. Tubing attached to chamber (b) dispensed the mixed solution via a peristaltic pump into the Biorad Protean® II XI casting plates slowly to avoid further mixing in the casting plates (Biorad Protean® II XI Cell – Instruction Manual).

To ensure a uniformly smooth interface at the gel surface, the gel was overlaid with a thin layer of water-saturated butanol and allowed to fully polymerize for several hours. Following removal of butanol and several rinses a stacking gel was cast on top of the separating gel. Typically gels were cast using 15 well combs for sample
loading or a single large well for experiments involving isoelectric focusing in the second SDS-PAGE dimension.

Polyacrylamide separating gels consisted of; Tris-HCl (pH 8.8) 375 mM; SDS 0.10% (w/v); acrylamide 7.5 % (w/v) in 7.5% gels, 15 % (w/v) in 15% gels or 19.9% (w/v) in 20% gels; bis-acrylamide 0.20 % (w/v) in 0.40% gels, 15 % (w/v) in 15% gels or 0.50% (w/v) in 20% gels; glycerol 0.70% (v/v) in 7.5% gels, 1.30 % (v/v) in 15% gels or 1.30% (v/v) in 20% gels; APS 2.2 mM; and TEMED 0.04% (v/v). Polyacrylamide stacking gels consisted of; Tris-HCl (pH 8.8) 125 mM; SDS 0.10% (w/v); acrylamide 4.50% (w/v); bis-acrylamide 0.12% (w/v); APS 0.13-0.26 mM; and TEMED 0.10 % (v/v). Tris-HCl (pH 8.8) and SDS were prepared as a stock solution at 4x concentration for lower gels (7.5% - 20%) and a separate stock for the stack. Acrylamide and bis-acrylamide prepared as a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide. Ammonium Persulfate was prepared as a 10% (w/v) (438 mM) stock as a daily stock.
Figure B.4. Gradient gel casting. Perspex™ chambers containing (a) 7.5% and (b) 20% poly-acrylamide solutions are connected in series. The polycrylamide solutions slowly combine in (b), reducing the percentage polyacrylamide over time. The combined solution is pumped into the gel casting plates resulting in a gradient of polyacrylamide, greater at the bottom than at the top.
**SDS-PAGE loading and running**

Gels were inserted in either a Biorad mini-PROTEAN electrophoresis module (BioRad, Australia) or a large format Biorad PROTEAN II XI cell. The electrophoresis module is filled with a SDS-PAGE “running buffer” containing Tris 25 mM, glycine 192 mM and SDS 0.1 % (w/v).

Protein samples were solubilized in an SDS sample buffer consisting of; Tris-HCl 25mM, glycine 30% (v/v) and SDS 1% (w/v) and bromophenol blue 0.01% (Laemmli, 1970) prior to loading.

Electrophoresis was carried out using the Biorad mini-PROTEAN module, run at a constant voltage of 200 V for one hour. Gels loaded in the Biorad PROTEAN II XI cell were run at 35 mAmps per gel for 4.5 hours or until the bromophenol blue dye front reach the bottom of the gel.
Isoelectric focusing

Premise

Proteins consist of both positively and negatively charged amino acids, the combination of which give individual proteins a characteristic inherent charge or isoelectric point (Pi). Isoelectric focusing separates proteins based on their isoelectric point. Samples are prepared in a non ionic sample buffer, often urea based, and electrophoresed in a 4% polyacrylamide matrix into which a pH gradient is established. In experiments conducted herein we made use of commercially available isoelectric focusing gels manufactured with an immobilized pH gradient (IPG) (BioRad, Australia). When a strong electrical current is place across the isoelectric focusing gel proteins migrate to the pH in the gel that corresponds to their isoelectric point at which their inherent charge is neutral (Fig B.5).

Modifications to a protein such as phosphorylation or oxidation usually only impart very small changes in molecular mass that may not be resolved using traditional SDS-PAGE, however, post-translational modifications that induce a charge bias to the protein can be effectively separated from their unmodified counterparts using isoelectric focusing. Following isoelectric focusing based on protein charge, proteins are further resolved along a 2\textsuperscript{nd} dimension (size) using standard SDS-PAGE to separated proteins based on mass.
Figure B.5. An immobilized pH gradient (IPG) strip containing a protein sample before and after isoelectric focusing. The protein sample (dashed lines) is initially distributed evenly along the IPG strip prior to isoelectric focusing (top). During isoelectric focusing (bottom), each protein migrates to its Pi (eg. pH > 4.1).
**IEF sample preparation**

Samples, either snap frozen single fibers or a crushed muscle homogenate, were prepared in rehydration buffer. The rehydration buffer contained; thiourea 2 M; urea 7 M; 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) 2% (non-ionic detergent) and bromophenol blue 0.005% and was stored as 500 µL aliquots at -80°C. Ampholytes, pH 3-10 0.05% (Biorad) and pH 4-7 1% (GE Healthcare), were added directly before use. The chaotropic agents urea and thiourea denature proteins and inactivate proteases, consequently no further attempt was made to inhibit proteases. To load the isoelectric focusing gels 345 µL of rehydration buffer, containing the protein sample was distributed evenly along the well of a rehydration tray and the exposed 18 cm pH 3-6 IPG strip laid, gel side down, onto the sample. Air bubbles were removed before overlaying the IPG strip with 2-3mL of mineral oil to prevent dehydration. The rehydration tray was covered and left overnight to allow complete loading of the sample.

Prior to isoelectric focusing the rehydrated IPG gel was drained of oil by placing on parafilm™ and placed gel side down in the PROTEAN IEF (Biorad, Australia) focusing tray. Lightly wetted 1 cm paper wicks were installed over each electrode to trap any residual salts. The strip was again covered with 2-3 ml of mineral oil and air bubbles removed.
Isoelectric focusing protocol

Following sample incorporation into the IEF strips a three-step method was used to effect isoelectric focusing of proteins: 1) 0-250 V rapid linear voltage ramp over 15 minutes; 2) 250-10 000 V ramp over 3 hours; 3) 10 000 V for a total of 60 000 V hours and 4) a hold step at 500 V.

Following isoelectric focusing the IPG strip was placed on parafilm™ to remove residual mineral oil followed by brief incubation in a series of buffers designed to enable SDS incorporation into proteins while removing the urea and thiourea and CHAPS. Step 1 involved a 10 minute equilibration in 6 ml of a solution containing urea 6 M, Tris-HCl 0.375 M (pH 8.8), SDS 2% and, glycerol 20% for 10 minutes. Step 2, 10 minute equilibration in 6 ml a solution containing urea 4 M, Tris-HCl 0.375 M (pH 8.8), SDS 2% and, glycerol 20% followed by briefly immersing in SDS-PAGE running buffer prior to loading onto a large format SDS-PAGE gel fitted with a preparatory well for 2nd dimension separation by SDS-PAGE (see above).
Western blot analysis

Premise

Western blot analysis was used to identify specific proteins that had previously been separated by SDS-PAGE or IEF and SDS-PAGE, then transferred and immobilized onto a nitrocellulose membrane. Proteins were visualized using polyclonal or monoclonal antibodies specific to proteins of interest and secondary antibody conjugated to the enzyme, horse-radish peroxidase, which reacts with luminol emitting light enabling detection of the proteins of interest using autoradiographic film.

Transfer of proteins to nitrocellulose

Following separation using SDS-PAGE, proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane. The Biorad Mini-Protean II transfer system was used for smaller gels, or a Biorad Transblot cell for larger gels. Proteins were transferred in a transfer buffer composed of; Tris 25 mM, glycine 192 mM and methanol 20% (v/v). The polyacrylamide gel and nitrocellulose were placed together, carefully removing all air space between. Filter paper is place on the outside as well as Scotch-Brite™ pads. The bundle is held together in a transfer cassette that fits vertically into the transfer system. The cassette is orientated with the nitrocellulose membrane towards the positive electrode of the transfer unit encouraging transfer of SDS coated, negatively charged proteins from the polyacrylamide onto the
nitrocellulose. The entire cassette was immersed in transfer buffer and cooled during the transfer process, either by a manufacturer-supplied ice pack or by conducting the transfer in a refrigerated cold room. Mini-Protean II transfers were run at 100 V for one hour while larger Transblot cell transfers were run at 30 mAmps for 16 hours. It is important to recognize that following transfer not all proteins were transferred equally, largely this is dependent on molecular weight. Consequently following transfer gels were also stained with coomassie brilliant blue R-250 which provided one with the opportunity to normalize protein load to the relative amount of myosin heavy chain remaining in the polyacrylamide gel.

**Incubation with primary and secondary antibody**

Nonspecific binding of primary and secondary antibodies to the nitrocellulose was prevented by incubating the nitrocellulose in 50 ml blocking solution (in mM); TBS (Tris-HCl (pH 7.4) 50, NaCl 150); Tween-20 0.05% (v/v); and nonfat milk powder 3% (w/v) for one hour.

Primary antibodies to Troponin C and light chain (LC$_{20}$) (MRCL3/MRLC2/MYL9) were diluted to 1:1000 and obtained from (Santa Cruz Biotechnology, USA) in a solution containing 10ml TBS, tween 0.05%, nonfat dried milk 1% solution. The nitrocellulose was then incubated in the antibody solution for one hour with constant agitation.
In order to visualize the primary antibody-bound protein a secondary anti-body conjugated to horseradish peroxidase (HRP) was used. The secondary antibody, specific for either rabbit IgG (TnC) or mouse IgG (LC$_{20}$), binds to the primary antibody and in the presence of a chemiluminescent solution, generates light detectible by X-ray film. Secondary antibody was diluted 1:10000 (rabbit IgG) or 1:5000 (mouse IgG) in TBS, 0.05% tween-20 solution and the nitrocellulose incubated for one hour, gently agitated.

**Chemiluminescence detection of specific proteins**

Chemiluminescent solution (Thermo Scientific, Super Signal West Pico™) was prepared immediately prior to application to the nitrocellulose membrane as per the manufactures instructions. Following application excess reagent was removed and the membrane covered in clear, commercial plastic wrap. In the dark autoradiographic film (medic grade X-ray film, AGFA) was exposed to the western blot to produce a series of exposure lengths ranging from 30 sec to 10 minutes. Film was developed on an AGFA CP1000 processor as per the manufacturers instructions.

**Data analysis**

Developed film and coomassie stained gels were scanned on a densiometric scanner (GS-710, Biorad) and optical density (OD) analyzed using Quantity One software (Biorad).
Optical density is the result of intensity of a given pixel in the digital scan. Quantity One detects the average OD for a selected area, multiplied by the area (OD*mm$^2$) and is referred to as a volume. To remove background signal, an area of film or gel that does not contain signal is selected and the resulting background volume subtracted from signals of interest.
Recombinant mutant TnC expression

Premise

Recombinant proteins used in the current study were a gift from Louise Brown’s laboratory (Macquarie University, Sydney). Nevertheless for completeness in the thesis the basic procedures are outlined below. *E coli* mediated recombinant protein expression was used to produce a wild type and mutant TnC containing a C84S substitute. Plasmid vectors containing the target gene were used as templates. Oligonucleotide primers containing the desired mutation were annealed to the parental DNA plasmid and extended and incorporated into new plasmid strands. The parental DNA was digested leaving only the new mutated plasmid strands. These strands were then transformed into competent cells and grown, producing the desired mutated protein (Fig B.6).
Figure B.6. Overview of site-directed mutagenesis using a QuikChange Mutagenesis II site directed mutagenesis kit. Step 1: Parental DNA plasmid containing the target protein (cTnC) was prepared. Step 2: Temperature cycling incorporated the mutagenic primer into polymerized circular mutated DNA plasmid strands. Step 3: The parental DNA plasmid was digested and the mutated DNA plasmid was transformed into competent cells. (Diagram modified from QuikChange Mutagenesis Site-Directed Mutagenesis Kit instruction manual, Revision A.01).
**Mutagenesis**

Single cysteine cTnC mutants, Cys35 and Cys84 were engineered by substituting the triplet of nucleotide bases encoding cysteine for a serine encoding triplet. Oligonucleotide primers containing the desired mutation for both the forward and reverse strands of the plasmid had previously been designed (Brown et al., 2002).

Mutagenesis was carried out in a pET-3d expression vector (Novagen), containing rat cTnC, using the Quickchange Mutagenesis II site directed mutagenesis kit (Stratagene) as per manufacturer’s protocol (Fig B.6).

Temperature cycling on a thermal cycler incorporates the mutagenic primer into polymerized circular plasmid strands. Briefly; the plasmid containing cTnC was denatured, the oligonucleotide primers containing the desired mutation were annealed to the plasmid, and a DNA polymerase extended and incorporated the mutagenic primer into new plasmid strands. To remove the nonmutated cTnC plasmid strands and ensure only mutated cTnC was expressed the parental DNA plasmid was digested with Dpn I. The Dpn I endonuclease was specific for the methylated and hemimethylated DNA of most strains of *E. coli* used as expression vectors, including pET-3d. All PCR was conducted by Dr Brown’s laboratory.
**Transformation and expression**

The cTnC plasmid was transformed into competent *Escherichia coli* BL21 (DE3) cells. *Dpn I* treated DNA were mixed with competent cells and incubated for 30 minutes on ice. The transform reactions were heat pulsed for 45 seconds at 42°C and placed on ice for 2 minutes. Preheated NZY+ broth, prepared as per the manufacturer’s protocol, was added to the transform reaction and incubated at 37°C for 1 hour. The transform reaction was then plated on agar plates containing ampicillin to select for cells containing the plasmid vector.

Protein expression was achieved through overnight cell growth at 37°C. Cells were harvested by centrifugation and suspended in Tris 50 mM (pH 7.5), DTT 1 mM, NaCl 100 mM and CaCl$_2$ 5 mM.

**Purification**

Cells were lysed using a french press at 15000 psi and centrifugation was used to remove cell waste. The supernatant was brought to CaCl$_2$ 10 mM (in the presence of Ca$^{2+}$ the hydrophobic surfaces become exposed) and dialyzed against the suspension buffer and loaded onto an equilibrated hydrophobic affinity interaction chromatography column, Phenyl Sepharose 6B (Pharmacia, GE Healthcare). Proteins bound non-specifically were removed using a salt wash (in mM: Tris 50 (pH 7.5), DTT 1, NaCl 200 and CaCl$_2$ 1) and a EDTA solution used to sequester Ca$^{2+}$ from TnC causing it to be release from the column (Tris 50 mM (pH 7.5), DTT 1 mM, NaCl 200 mM and EDTA 10 mM). Samples were assessed by UV spectroscopy.
Absorption peaks obtained from UV spectroscopy correlate to bond types present and was used to confirm correct tertiary structure. Nucleic acid contaminants were removed using a DEAE Sephadex column (Sigma-Aldrich) and a salt gradient (NaCl 0.35 M-1 M). Purity of cTnC and cTnC mutants was confirmed with SDS-PAGE (Fig B.7). Samples were lyophilized and stored at -20°C. Lyophilized recombinant TnC was dialyzed against HEPES 25 mM (pH 7.4) prior to use. Protein concentration following dialysis was determined using a Pierce BCA assay (Thermo Scientific, USA).
Figure B.7. Purity of cTnC and cTnC mutants confirmed with coomassie brilliant blue stained SDS-PAGE. DTT was the only reducing agent included in the SDS-PAGE samples, at concentrations of 0, 10 mM and 25 mM. Samples included in the gel are wild-type cTnC (WT) and TnC C84 (C84S). Dimerisation was observed in no DTT samples (*). Modified from an image provided by collaborators, Dr Louise Brown and James Cooke at the Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia.
Acknowledgements

Recombinant wild-type and mutant cTnC expression and purification was carried out by collaborators, Dr Louise Brown and James Cooke at the Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia.
Sequential effects of GSNO and H$_2$O$_2$ on the Ca$^{2+}$-sensitivity of the contractile apparatus of fast- and slow-twitch skeletal muscle fibers from the rat

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STATEMENT OF CONTRIBUTION

This chapter in whole was published in the American Journal of Physiology – Cell Physiology, vol 296 (2009): C1015-C1023. Timothy Spencer and Dr Giuseppe Posterino jointly authored the paper.

Dr Posterino gives permission for the paper to be included in the thesis. It is acknowledged that in the conceptualization, spectrophotometric experimentation and analysis and editing of the paper Dr Posterino contributed 25 % of the paper.

The remaining work (75 %) is to be attributed to Timothy Spencer.

Dr Giuseppe Posterino

Timothy Spencer

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1152/ajpcell.00251.2008
Myofibril basis for oxidative dysfunction in skeletal muscle
Abstract

It is evident the redox state can potentiate or inhibit cellular muscle function. Our previous studies in demembranated fast-twitch skeletal muscle fibers have shown that, the oxidant H$_2$O$_2$ increases Ca$^{2+}$-sensitivity, while NO decreases the Ca$^{2+}$-sensitivity impairing contraction. We chose to investigate the myofilament basis for the decline in Ca$^{2+}$ sensitivity in the fast-twitch skeletal muscle following NO exposure.

Muscle myography was used to analyse the functional consequence of NO exposure on single demembranated fast-twitch muscle fibers. Isoelectric focusing and SDS-PAGE proteomic analysis coupled with western blot analysis identified modified muscle fiber proteins following NO exposure. Molecular substitution of endogenous TnC with recombinant cardiac/slow WT-TnC and cardiac/slow C84S TnC, containing a non-oxidisable serine residue at position 84, identified the functional myofilament substrate of NO mediated Ca$^{2+}$ desensitisation.

In demembranated, fast-twitch skeletal muscle, the NO donor GSNO reduced the Ca$^{2+}$-sensitivity of the muscle fiber. Proteomic analysis showed that among a number of muscle fiber proteins, myosin light chains and TnC were modified in the presence of GSNO. Molecular substitution of endogenous TnC with cardiac/slow WT-TnC had a similar sensitivity to NO as fast-twitch skeletal muscle. In contrast, substitution with C84S TnC was insensitive to NO. These results indicate that although multiple muscle fiber proteins are modified following NO exposure, C84S TnC is the
functional substrate resulting in the GSNO mediated decline in muscle fiber Ca^{2+}-sensitivity.
Introduction

Contraction of cardiac and skeletal muscle is a highly regulated process involving; regulation of extracellular and sarcoplasmic reticulum (SR) Ca\(^{2+}\) entry, generation of ATP, and Ca\(^{2+}\) dependent conformational changes of structural, regulatory and motor proteins. It is becoming increasingly evident that redox state leading to post-translational modifications of structural proteins, enzymes and ion channels can cause activation or inhibition of cellular function (Andrade et al., 1998a, Jackson, 2008, Kelly et al., 1996). Cells are able to maintain their redox balance despite the generation of reactive oxygen (ROS) and reactive nitrogen species (RNS) primarily due to the production of endogenous anti-oxidants and reductants that convert these molecules to harmless electron stable molecules or remove modifications caused by an oxidative reaction. Therefore it follows that molecular, cellular and systems function can be regulated by an excess of oxidant or reductant.

The physiological response to ischemia as a consequence of coronary artery disease, heart failure, claudication or during heavy exercise in skeletal muscle is for the vascular endothelium to release the potent vasodilator, nitric oxide (NO). Interestingly, with prolonged ischemia tissue damage occurs leading to an inflammatory response and subsequent release of large amounts of NO from inflammatory cells, neutrophils (Jordan et al., 1999).

In addition, studies have demonstrated that the administration of exogenous NO donors to functional cardiac and skeletal muscle preparations have a negative effect
on the contractile function. Using the diaphragm muscle mixed fiber type, Kobzik et
al., (1994a) have shown that nitric oxide synthase (NOS) inhibitors such as nitro-L-
arginine improve contractile function including the force-frequency relationship, and
that these improvements in contractile function could be reversed by exogenous NO
donors. Similarly, in isolated, electrically stimulated cardiomyocytes, the application
of an NO donor or superfusion with an NO solution attenuated contraction (Brady et
al., 1993). The suggestion has been that NO can detrimentally affect one or more
elements of excitation-contraction coupling in muscle. The control of cytoplasmic
Ca$^{2+}$ concentration by the ryanodine receptor (RyR) has been a focus of a number of
studies (Aracena et al., 2003). In whole muscle preparations the functional impact of
oxidation on the RyR is a significant reduction in Ca$^{2+}$ release and diminished
contractile function (Andrade et al., 1998a, Posterino and Lamb, 1996). In addition,
proteins within the muscle fiber may also be oxidized and contribute directly to
reduced muscle function.

Several components of the muscle fiber have the potential to be, or have been shown
to be modified when in an oxidising environment (Dalla Libera et al., 2005).
However, the majority of studies have either focussed on associated changes ie.,
relatively indirect assessment of altered function, or biochemical changes. Exposure
of permeablized rabbit psoas single muscle fibers to an NO donor such as SNAP and
NEM has been shown indirectly to reduce ATP hydrolysis by the myosin ATPase
through analysing the conversion of a fluorescent NADH to nonfluorescent NAD$^+$
(Perkins et al., 1997). The functional consequence of NO exposure was only
indirectly assessed. Spin labelled myosin ATPase in a rabbit psoas muscle fiber,
showed that when over 75% of the SH-1 sites are modified a significant decrease in
contractile function was observed (Crowder and Cooke, 1984). The light chains of myosin (LC\textsubscript{20}) have also been identified as a possible target of oxidation. Light chains reconstituted with myosin, oxidised following 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) exposure, form intramolecular disulfide bonds (Wolff-Long et al., 1993), however, the study used an incomplete myofilament preparation precluding investigation of the functional importance of LC\textsubscript{20} oxidation. Actin in its globular form has several cysteine residues that can be oxidised, however, when polymerised into a functional actin filament there is less oxidation or it is abolished (Liu et al., 1990). Therefore, although many studies have shown the capacity of myofilament proteins to be oxidised, few experiments have directly linked contractile dysfunction with oxidation of a specific myofilament protein.

Different muscle fiber types display isoform specific differences in many myofilament proteins, but nevertheless share similar Ca\textsuperscript{2+}-dependent myofilament regulation mediated by the troponin complex (Bottinelli and Reggiani, 2000). The functional similarity of skeletal and cardiac muscle myofilament proteins has allowed for substitution of native skeletal muscle proteins with cardiac isoforms, while maintaining myofilament function (Davis and Tikunova, 2008, Moss et al., 1986b, Moss et al., 1991).

In the current study we made use of a simplified, demembranated fast-twitch muscle fiber preparation to enable the identification of the functional importance of NO mediated oxidative stress on the contractile apparatus independent from other components of excitation-contraction coupling. We have used functional force Ca\textsuperscript{2+}
measurement, protein substitution experiments using oxidisable and non-oxidisable (at Cys84) forms of TnC and biochemical analysis of myofilament proteins to investigate the functional, myofilament basis for GSNO mediated fast-twitch skeletal muscle dysfunction and the role of specific cysteine residues on cardiac TnC.
Methods

Muscle preparation

All experiments were performed according to the guidelines and with approval of the University of Adelaide Animal Ethics Committee. Male rats (Sprague-Dawley, approximately 400g) were euthanized in a CO₂ chamber. The EDL muscles were excised, immersed under paraffin oil, and kept on ice. Under a dissecting microscope, single fibers were isolated from whole muscle using fine forceps and the sarcolemmal membrane removed. The single fiber was then mounted between a force transducer (2kHz resonance frequency; AME801, SensoNor, Horten, Norway) and stationary pin and stretched to 120% of its resting length. The adjusted length and diameter were then remeasured. Following from Posterino and Lamb (1996) the fiber was immersed in a Perspex™ bath containing a K-EGTA (in mM: K⁺, 126; Na⁺, 36; EGTA²⁻, 50; total ATP, 8; creatine phosphate (CP), 10; total Mg²⁺, 10.3; HEPES, 90; pH 7.10; pCa (-log₁₀ [Ca²⁺]), <9.0) solution containing 2% Triton-X 100 for 10 minutes to solubilize the remaining membranous compartments.
Functional analysis

*Ca\(^{2+}\) activation of the contractile apparatus*

Single fibers were mechanically skinned using forceps and were subsequently chemically skinned in K-EGTA solution containing 2% Triton-X 100 for 10 min to destroy all remaining membranous compartments. The fiber was then washed free of cytosolic proteins and residual membranous compartments using K-EGTA solution for 2 min. Prior to Ca\(^{2+}\) activation fully demembranated single muscle fibers were equilibrated in K-HDTA (a non-chelating analog of EGTA) solution which contained (in mM); K\(^+\) 126, Na\(^+\) 36, HDTA 50, total ATP 8, creatine phosphate 10, total Mg\(^{2+}\) 8.5, EGTA 1, HEPES 90, pH 7.10, pCa (-log\(_{10}\)[Ca\(^{2+}\)]) < 9.0. The force-pCa relationship was determined by activation of the fiber in Ca\(^{2+}\)-EGTA solutions (in mM); K\(^+\) 126, Na\(^+\) 36, EGTA 50, total ATP 8, creatine phosphate 10, total Mg\(^{2+}\) 8.1, HEPES 90 mM, pH 7.10, Ca\(^{2+}\) 50, pCa 4.5. To effect full relaxation of the contractile apparatus, fibers were fully relaxed in a heavily buffered K-EGTA solution. Both the K-EGTA and Ca\(^{2+}\)-EGTA solutions were mixed in a series of ratios to give solutions with a pCa range of between 7.0 and 4.5. Fibers were activated through a sequence of these solutions ranging in pCa from < 9 to 4.5 to establish the full Ca\(^{2+}\)-dependent force (force-Ca\(^{2+}\)) relationship. Previous experiments have determined the free [Ca\(^{2+}\)] for ratios used (Spencer and Posterino, 2009).

The experimental protocol with GSNO involved three successive control activation sequences fibers followed by exposure to GSNO (1mM for 2 min in a Ca\(^{2+}\) free K-
HDTA solution. Fibers where then washed in a K-HDTA solution for 2 min followed by generation of the force-pCa relationship using fixed [Ca$^{2+}$] solution for pCa 7.0 - 4.5.

The nitric oxide donor, GSNO was made fresh every hour as a 10 mM stock and further diluted, to 1 mM. Previous experiments have determined the amount of NO liberated from GSNO 1 mM in solution at 23 °C (Spencer and Posterino, 2009). Specifically the peak NO concentration immediately upon dissolving GSNO 1 mM in solution was 14 μM with a half-life of approximately 10 mins. At approximately 20 mins after addition, a low level NO concentration of between 1-4 μM was elicited for a further 40 mins. Experiments were conducted at the stage where the NO concentration was ranging from 1-4 μM. The reducing agent Dithiothreitol (DTT) was made as a 1 M stock and then further diluted to the required concentration during experiments. All chemicals were obtained from Sigma.

**TnC extraction and reconstitution with recombinant TnC**

Following an initial force-pCa series in single skinned muscle fiber troponin C was extracted using a modified protocol from Moss et.al (1986). Briefly, fibers were incubated in a low salt solution (in mM: Tris 20, EDTA 5, DTT 1) for 1 hour. The extraction solution was retained, frozen and lyophilized for later analysis of extracted proteins. The fiber was next washed in K-HDTA solution for 2 min followed by a second Ca$^{2+}$ series for pCa 7.0 – 4.5. Fibers were subsequently incubated in K-EGTA solution with recombinant TnC (~1 mg/ml) and DTT (25 mM) for 1 hour. A high
DTT concentration was required to prevent dimerisation of recombinant TnC (see Fig B.7 in General Methods). Following reconstitution of recombinant TnC or C84S TnC the force-Ca\textsuperscript{2+} relationship was again redetermined. A subpopulation of fiber, reconstituted with either WT cardiac/slow or C84S cardiac/slow TnC were exposed to GSNO (1 mM) in K-HDTA for 2 min prior to repeating the force-Ca\textsuperscript{2+} relationship.

**Data analysis**

The activation sequence within a fiber was individually analysed. Sub-maximal responses were normalized to the maximum Ca\textsuperscript{2+}-activitated force produced in the initial Ca\textsuperscript{2+} activation sequence. The data obtained from each pair of activation sequences was then averaged and plotted using GraphPad Prism v4.01 (GraphPad Software, San Diego, CA). The force-Ca\textsuperscript{2+} data were fitted with a modified Hill equation. From the resulting curve the pCa\textsubscript{50} (the pCa at which 50% maximum force is produced) was determined as a measure of Ca\textsuperscript{2+}-sensitivity.

The results are presented as means ± SEM for a sample of \(n\) fibers. Statistical significance was examined using either a student’s unpaired t-test or repeated measures one-way analysis of variance where appropriate with a significance level of \(P<0.05\).
Proteomic analysis

Isoelectric focusing

Fast-twitch muscle homogenate was prepared by snap freezing EDL under liquid nitrogen and homogenized in liquid nitrogen and immobilised pH gradient (IPG) rehydration buffer.

Fast-twitch skeletal muscle homogenates were prepared for isoelectric focusing (IEF) by dilution of ~1 mg (total protein) in 315 μL immobilised pH gradient (IPG) rehydration buffer containing urea 7 M, thio urea 2 M, CHAPS 2%, bromophenol blue 0.0001%, 3-10 pH carrier ampholyte 0.05% (Bio-Rad) and 4-7 pH carrier ampholyte 1% (GE Healthcare). Prior to dilution in 315 μL IPG rehydration buffer GSNO 1 mM or DTT 10 mM was added to the muscle homogenate for 2 min. Isoelectric focusing was carried out according to the manufacture’s protocol (Bio-Rad). Immobilised pH gradient strips (18cm pH 3-10 linear gradient, Bio-Rad) were rehydrated overnight at room temperature. Isoelectric focusing was carried out using a PROTEAN IEF cell (Bio-Rad) and included a 15 min 250 V first step to remove salts, a second voltage ramp from 250-10 000 V over 3 hours and a final 10 000 V focusing step for 60 000 Vhours. Fully focused proteins were placed on a 500 V hold cycle.
**Second-Dimension SDS-PAGE**

Prior to running the second dimension SDS-PAGE, IPG strips were equilibrated in SDS-containing buffers in a protocol modified from the manufacturer’s instructions (Bio-Rad). Briefly, IPG strips were initially equilibrated in buffer containing; urea 6 M, Tris-HCl 0.375 M (pH 8.8), SDS 2% and glycerol 20% for 10min. A second equilibration in buffer containing; urea 4 M, Tris-HCl 0.375 M (pH 8.8), SDS 2% and glycerol 20%, iodoacetamide 2.5% (w/v) for a further 10min. Prior to loading the IPG strip onto the SDS-PAGE, the IPG strip was placed in SDS running buffer containing Tris 25 mM, glycine 92 mM and SDS 0.1 % (w/v).

IPG strips were embedded in a 5% acrylamide stacking gel and proteins resolved by 7.5 - 20% gradient SDS-PAGE on a large format Biorad PROTEAN II XI module (Biorad, Australia). Samples were electrophoresed at 35 mAmp per gel for 4.5 hours. Gels were either protein stained in coomassie blue overnight and excess stain was removed by washing in 10% acetic acid, or for western blot analysis, transferred to 0.2 µm nitrocellulose (Biorad) using a Biorad Mini PROTEAN II transfer unit as 100 V for 1 hour or a large format Biorad Transblot apparatus at 100 V for 3 hrs in SDS-free transfer buffer composed of; Tris 25 mM, glycine 192 mM and methanol 20% (v/v).
Western blot analysis of TnC and LC$_{20}$

Following transfer of proteins to nitrocellulose, the western blots were blocked with 3% non-fat dried milk powder (NFDM) in TBS (in mM: Tris-HCl (pH 7.4) 50, NaCl 150) with Tween 0.05% (TBS-T) for 1 hour to minimise non-specific antibody binding. Following three 5 min washes in TBS-T western blots were incubated with the primary antibody, either anti-TnC (H-110) (1:1000 dilution, Santa Cruz) or anti-LC$_{20}$ (1:1000 dilution, Santa Cruz) in 1% NDFM in TBS-T for 1 hour. Western blots were again washed three times in TBS-T, to remove antibody not specifically bound to ligand, prior to incubation with the secondary antibody; anti-rabbit IgG-horseradish peroxidase-conjugated (1:10000 dilution, Santa CruZ) or anti-mouse IgG-horseradish peroxidase-conjugated (1:5000 dilution, Santa Cruz), in TBS-T for 1 hour. Three 5 min wash steps to remove non-specifically bound secondary antibody preceded the exposure to enhanced chemiluminescence reagents (Thermo Scientific). Luminescent signals were detected using autoradiographic film (AGFA).

Recombinant protein expression

Mutagenesis was carried out in a pET-3d expression vector (Novagen) containing rat cTnC using the Quickchange Mutagenesis II site directed mutagenesis kit (Stratagene) as per manufacturer’s protocol. Single cysteine cTnC mutants, Cys35 and Cys84 were engineered by removing native cysteine residues C84S and C35S, respectively (Brown et al., 2002).
Cardiac TnC plasmid was transformed into competent *Escherichia coli* BL21 (DE3) cells. Protein expression was achieved through overnight growth of transfected cells on a rotatory shaking platform at 37 °C. Cells were harvested by centrifugation and the pellet suspended in Tris 50 mM (pH 7.5), DTT1 mM, NaCl 100 mM and CaCl$_2$ 5 mM. Cells were lysed using a French Press at 15000 psi and centrifugation was used to remove cell debri. The supernatant was brought to 10 mM CaCl$_2$ and dialysed against the suspension buffer and loaded onto an equilibrated hydrophobic interaction chromatography Phenyl Sepharose 6B column (Pharmacia, GE Healthcare). Proteins bound non-specifically were removed using a Ca$^{2+}$ containing wash buffer (in mM: Tris 50 (pH 7.5), DTT 1, NaCl 200 and CaCl$_2$ 1). Sequestration of Ca$^{2+}$ using EDTA containing elution buffer (Tris 50 mM (pH 7.5), DTT 1 mM, NaCl 200 mM and EDTA 10 mM) enabled elution of TnC. Fractions containing TnC were assessed using UV spectroscopy. Nucleic acid contaminants were removed using a DEAE Sephadex column (Sigma-Aldrich) and a (NaCl 0.35 - 1 M) salt gradient. Purity of cTnC and cTnC mutants was confirmed with SDS-PAGE (see Fig B.7). Samples were lyophilized and stored at -20°C. Lyophilized recombinant TnC was dialysed with HEPES 25mM (pH 7.4) prior to use. Protein concentration following dialysis was determined using the Pierce bicinchoninic acid protein assay (Thermo Scientific).
Results

Ca$^{2+}$-sensitivity of fast-twitch skeletal muscle

Exposure of skinned fast-twitch skeletal muscle to the nitric oxide donor GSNO (1 mM) for 2 min, significantly decreases the Ca$^{2+}$-sensitivity of the contractile apparatus (initial 6.26 ± 0.02, GSNO treated 6.18 ± 0.01 n=6) without significantly decreasing the maximal force at pCa 4.5 (Fig D.1). Previous work has demonstrated that this change in Ca$^{2+}$-sensitivity can be reversed by incubation in K-HDTA solution containing DTT (10 mM) for 20 min (Spencer and Posterino, 2009)(Chapter 3 Fig C.1) .
Figure D.1. GSNO treatment decreases the Ca$^{2+}$-sensitivity of demembranated fast-twitch skeletal muscle fibers. The force-Ca$^{2+}$ relationship was determined in demembranated fast-twitch muscle fibers. Muscle fibers were subsequently exposed to GSNO (1 mM) for 2 min in K-HDTA solution and the force-Ca$^{2+}$ relationship examined. Exposure to GSNO significantly reduced the Ca$^{2+}$-sensitivity of the demembranated, fast-twitch skeletal muscle fiber (initial (■) EC$_{50}$ pCa 6.26 ± 0.02, GSNO treated (□) 6.18 ± 0.01 n=6). (*) P <0.05.
GSNO mediated modification of muscle fiber proteins

Homogenates of EDL exposed to DTT (10mM) or GSNO (1 mM) were analysed using 2-D PAGE coomassie brilliant blue R250 staining, followed by western blot analysis of TnC and LC$_{20}$. Coomassie blue staining of the SDS-PAGE gradient gel identified several altered protein spots. Western blot analysis of TnC identified a single spot for TnC was observed in the DTT reduced sample while following GSNO (1mM) for 2 min a shift in isoelectric point was seen in a proportion of TnC with a second distinct spot being identified, indicating a post-translational modification has occurred. The Western blot was subsequently probed with an antibody to LC$_{20}$. A similar shift the isoelectric point of LC$_{20}$ was observed with an altered proportion of LC$_{20}$ among three visible spots in the presence of GSNO, not DTT (Fig D.2).
Figure D.2. 2-Dimensional electrophoresis followed by western blot analysis of TnC and LC$_{20}$ in reduced (DTT) and GSNO treated skeletal muscle. Exposure of the fast-twitch muscle homogenates to GSNO (1 mM) resulted in a shift in isoelectric point of (b) TnC in comparison to DTT (10 mM) reduced muscle (a). The nitrocellulose was reprobed for LC$_{20}$. Western blot analysis of the LC$_{20}$ also identified an altered spot pattern of (d) the GSNO (1 mM) treated muscle in comparison to (c) DTT (10 mM) reduced. Data represents 1 of 3 independent experiments.
Substitution of native troponin C with recombinant troponin C

Following a 10 minute incubation in a low salt extraction buffer, the maximum force in single skinned muscle fibers was reduced to $48.0 \pm 3.4\%$ of the initial maximum. Incubation for a further 1 hour in K-EGTA solution with DTT (25mM) with recombinant TnC (~1 mg/ml) partially restored the maximum force (pCa 4.5). Upon incubation with wild-type TnC, maximum force was restored to $69.3 \pm 3.5\%$ of the initial maximum force prior to TnC extraction. Incubation with TnC C84S restored force from $41.3 \pm 2.1\%$ after TnC extraction to $61.8 \pm 2.2\%$ after reconstitution. The restoration of force suggests successful reconstitution of the myofilament with both wild-type and C84S mutant recombinant TnC.

Western blot analysis identified that low salt extraction buffer removed some TnC from the demembranated single muscle fibers but did not remove LC$_{20}$ (Fig D.4). Ratio analysis of TnC vs LC$_{20}$ is consistent with the removal of TnC (demembranated control: 0.73, following TnC extraction: 0.36 n=1) Taken together these data indicate that low salt extraction buffer facilitates partial removal of TnC consistent with observed changes in force (Fig D.3).
Figure D.3. The role of native and cardiac WT TnC and cardiac C84S TnC, insensitive to oxidation of C84S, in mediating Ca2+ dependent activation of force in demembranated skeletal muscle fibers. (a) Force Ca^{2+} relationship of: demembranated fast-twitch skeletal muscle fibers (■), demembranated muscle fibers following extraction of TnC (▲), and demembranated muscle fibers following constitution of TnC using WT cardiac TnC (□). (b) Force Ca^{2+} relationship of: demembranated fast-twitch skeletal muscle fibers (■), demembranated muscle fibers following extraction of TnC (▲), and demembranated muscle fibers following constitution of TnC using C84S cardiac TnC (△).
Table D.1. Changes in the pCa$_{50}$ of demembranated muscle fibers prior to and following recombinant TnC substitution.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>TnC extracted</th>
<th>Recombinant TnC</th>
<th>GSNO</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT (EC$_{50}$)</strong></td>
<td>6.05 ± 0.02</td>
<td>5.89 ± 0.04 *</td>
<td>6.01 ± 0.03**</td>
<td>5.87 ± 0.03#</td>
<td>7</td>
</tr>
<tr>
<td><strong>C84S (EC$_{50}$)</strong></td>
<td>5.96 ± 0.02</td>
<td>5.79 ± 0.03 *</td>
<td>5.86 ± 0.02**</td>
<td>5.82 ± 0.03</td>
<td>5</td>
</tr>
</tbody>
</table>

The table shows the EC$_{50}$ values of demembranated muscle fibers initially. Following TnC extraction the EC$_{50}$ is significantly reduced (*). Recombinant WT and C84S TnC was incorporated following native TnC extraction, significantly increasing the EC$_{50}$ (**). Exposure of demembranated muscle fibers with recombinant TnC incorporated to GSNO significantly reduced the EC$_{50}$ of WT (#) but not C84S recombinant TnC.
Figure D.4. Western blot analysis of demembranated single fibers before and following extraction of TnC. Western blot analysis using anti-TnC identified TnC in the demembranated single fibers following TnC extraction, as well as the low salt extraction buffer. Western blot analysis with anti-LC$_{20}$ identified LC$_{20}$ present in demembranated single fibers and demembranated single fibers following TnC extraction, but no LC$_{20}$ in the extraction buffer. Ratio analysis of TnC vs LC$_{20}$ is consistent with the removal of TnC (demembranated single myofibril: 0.73, TnC extracted myofibril: 0.36 ).
The role of C84 TnC in muscle fiber Ca\(^{2+}\)-sensitivity

Muscle fibers in which native TnC had been extracted and replaced with WT cardiac/slow TnC demonstrated a decrease in Ca\(^{2+}\)-sensitivity following exposure to GSNO (1 mM) for 2 min compared to control muscle fibers, in which TnC had been substituted but no GSNO added (untreated EC\(_{50}\) 6.01 ± 0.03 n=7, GSNO treated 5.87 ± 0.03 n=5) (Fig D.5.a). The maximum force produced by treated muscle fibers was not significantly different from untreated controls (untreated 69.9 ± 3.5% n=7, GSNO treated 64.0±3.6% n=5). In contrast muscle fibers containing the C84S mutant TnC showed no significant decrease in Ca\(^{2+}\)-sensitivity following exposure to GSNO (1 mM) for 2 min (untreated EC\(_{50}\) 5.86 ± 0.02 n=6, GSNO treated 5.82 ± 0.03 n=5) (Fig D.5.b). The maximum force of treated C84S muscle fibers was not significantly different from untreated C84S fibers (untreated 62.8 ± 2.2% n=6, GSNO treated 62.4 ± 4.1% n=5). The lack of change in Ca\(^{2+}\)-sensitivity in reconstituted muscle fibers containing C84S TnC suggests NO mediated Ca\(^{2+}\)-sensitivity changes are due, at least in part, to modification of the cysteine residue of TnC.
Figure D.5. Mutation of cysteine 84 of recombinant troponin C to a non-oxidisable serine prevents the GSNO mediated reduction in muscle fiber Ca\textsuperscript{2+}-sensitivity. Demembranated, fast-twitch skeletal muscle fibers following recombinant troponin C substitution. Following substitution of endogenous troponin C with recombinant cardiac/slow troponin C the muscle fiber was either reassessed for the second force-Ca\textsuperscript{2+} relationship (untreated) or treated with GSNO (1 mM) for 2 min. (a) The Ca\textsuperscript{2+}-sensitivity of muscle fibers containing recombinant troponin C decreased following exposure to GSNO (1 mM) (□) compared to untreated recombinant TnC but without GSNO exposure (■) (untreated EC\textsubscript{50} 6.01 ± 0.03, n=7, GSNO 5.86 ± 0.03 n=5). (b) Substitution of mutant, cardiac/slow C84S troponin C into muscle fibers showed no significant change in Ca\textsuperscript{2+}-sensitivity following exposure to GSNO (1mM) (▲) compared to muscle fibers containing C84S TnC but not exposed to GSNO (△) (untreated EC\textsubscript{50} 5.86 ± 0.02, n=6, GSNO 5.82 ± 0.03, n=5). (*) P<0.05.
Discussion

Muscle weakness associated with ischemic conditions may not be limited to metabolic constraints but also myofilament dysfunction mediated by nitric oxide. Ischemic conditions often cause endothelial cells to increase NO production causing vasodilation. Prolonged ischemia activates inflammatory cells such as neutrophils leading to a further, release of NO.

There is clear evidence that the addition of high levels of exogenous NO is associated with an impairment of skeletal and cardiac muscle contraction (Gath et al., 1996, Kobzik et al., 1994a, Morrison et al., 1996). It is generally recognised that oxidative modification may affect ion channels involved in action-potential propagation and ion gradients, the coupling of L-type Ca\(^{2+}\) channels to the RyR of the SR, the RyR and SERCA2 pumps that control cytoplasmic Ca\(^{2+}\) concentration and mitochondrial proteins involved in metabolism (Lamb, 2002, Jackson, 2008, Zima and Blatter, 2006, Stamler and Meissner, 2001, Marechal and Gailly, 1999). However, in this study the focus has been the myofilament basis of NO mediated dysfunction. Our study not only illustrates a muscle fiber-dependent functional change in Ca\(^{2+}\)-sensitivity, but using molecular substitution, we have identified that TnC is the regulatory sensor mediating muscle fiber sensitivity to GSNO.

While the changes in Ca\(^{2+}\)-sensitivity have been observed using other NO donors (Perkins et al., 1997, Spencer and Posterino, 2009, Heunks et al., 2001, Andrade et al., 1998b), the same decline has not been observed with the oxidant H\(_2\)O\(_2\) (Posterino and Lamb, 1996, Andrade et al., 1998a, Spencer and Posterino, 2009). Although we
have identified the specific substrate of oxidation we have not yet identified the endogenous reductant that would normally reverse this process following restoration of the normal redox environment.

Previous studies have identified that the myosin ATPase, actin, troponin I and T, as well as light chains of myosin (LC20) are all substrates of oxidation (Crowder and Cooke, 1984, Perkins et al., 1997, Labugger et al., 2000, Dalla Libera et al., 2005, Wolff-Long et al., 1993). Specifically, Crowder et al. (1984) found that when 75% or more of the SH-1 sites of myosin were spin-labelled there was an observable change in contractile function, however, the authors concluded that in order to achieve a 75% saturation of SH-1, labelling was likely to become non-specific and that the change in contractile function was attributed to non-specific targets. Other myofilament proteins including globular actin have been shown to be oxidised but when placed in association with either myofilament proteins, or in the case of G-Actin as polymeric F-Actin, are no longer a substrates of oxidation (Liu et al., 1990). Consequently, although several direct biochemical targets have been identified, physiologically they are not likely functional targets of oxidation nor would they be expected to mediate muscle fiber oxidative dysfunction.

Troponin C was successfully extracted from functional skeletal muscle using a classical approach of low salt buffer extraction (Moss et al., 1985), reducing the hydrophobic interactions of myofilament associated proteins. The passive extraction technique lead to a significant drop in Ca^{2+}-sensitivity and force, consistent with the loss of native TnC. The fact that not all force was lost illustrates that extraction was
likely incomplete and is consistent with our biochemical analysis. However upon addition of exogenous WT TnC force was significantly improved suggesting that both extraction and replacement of TnC was successful and is consistent with the work of Moss et al. 1986. The partial restoration of function, lost after the extraction, demonstrates that despite structural differences cardiac/slow TnC (Fig A.6) functions similarly to native fast-twitch TnC. Incomplete restoration of force may suggest that not all the lost TnC is replaced. Without the presence of chaperones or buffers that significantly modulate ionic strength incomplete restoration of TnC is not surprising particularly if one considers that the tight packing of myofilament proteins are likely to provide considerable steric hindrance to impede the reincorporation of TnC protein into the myofilament. However, SDS-PAGE, coomassie brilliant blue and silver staining analysis of the extraction buffer was unable to detect any significant loss of other myofilament proteins other than TnC. Other groups have successfully achieved complete extraction of TnC using chelators such as trifluoperazine to remove Troponin, however, this may also remove associated LC_{20} (Trybus et al., 1994). Despite these limitations with the biochemical analysis our functional data provides compelling evidence that demonstrates 1) the loss of function following TnC extraction and 2) the restoration of function following TnC replacement and 3) insensitivity to GSNO following replacement of extracted C84S TnC but not following WT TnC.
The fact that demembranated single muscle fibers reconstituted with mutant C84S TnC were insensitive to GSNO-mediated reduction in muscle fiber Ca\(^{2+}\)-sensitivity despite the oxidation of other proteins, including LC\(_{20}\), clearly demonstrates that the reduction in Ca\(^{2+}\)-sensitivity following GSNO exposure is mediated by TnC.

The GSNO-mediated reduction in Ca\(^{2+}\)-sensitivity by muscle fibers containing recombinant WT TnC further demonstrates that recombinant cardiac TnC is functioning in a manner similar to native fast-twitch skeletal TnC and is consistent with previous substitution experiments (Moss et al., 1986b, Moss et al., 1991). Cysteine residue along with the amino acids methionine and tyrosine are also potentially susceptible to oxidation. However, the fact that muscle fibers were insensitive to GSNO in the presence of C84S but not WT TnC suggests other oxidisable amino acid residues are; 1) not available, 2) not accessible, or 3) not functionally relevant to the GSNO mediated reduction in Ca\(^{2+}\)-sensitivity. The Cys84 residue of cardiac TnC sits at the low affinity Ca\(^{2+}\) binding site (Tikunova et al., 2010) making it an ideal target to modulate Ca\(^{2+}\)-sensitivity as alterations may further reduce the Ca\(^{2+}\)-binding affinity. Although cardiac TnC has a second cysteine residue at position 35 our C84S TnC data suggests that it is either not available or not regulatory. In fast-twitch TnC there is a single cysteine located at position 98 near but not in the low affinity Ca\(^{2+}\) binding region. The single cysteine residue at position 98 may be the site of oxidation mediating a decline in Ca\(^{2+}\)-sensitivity following GSNO exposure. Although Cys98 is not situated in the low affinity binding site comparison of species specific homologs of TnC has demonstrated that as little as a 5 amino acid difference, distal from the low affinity binding site, can impart significant changes to Ca\(^{2+}\) binding affinities (Gillis et al., 2007). Future studies aimed at investigating the
role of residue cys98 in skeletal muscle TnC will evaluate this possibility. However, taken together these data suggests that the oxidative modification of the cys84 residue, and not other residues of TnC or other proteins are responsible for the decrease in Ca\(^{2+}\)-sensitivity in fibers exposed to GSNO. An s-nitrosylation (Hess et al., 2005, Hess et al., 2001), of the cys84 residue is perhaps the most likely modification which will be explore by future mass-spectroscopy analysis.

**Future directions and implications**

The manner in which these studies were conducted also has several important implications to cardiac muscle in which similar single fiber experiments less easily conducted due to limitations in the ability to isolate individual cardiac fibers. Others have investigated cardiac muscle function using rat trabeculae (~100\(\mu\)m diameter) that are far larger in comparison to rat skeletal muscle fibers (~50\(\mu\)m diameter), requiring longer perfusion time (Kentish et al., 1986, Konhilas et al., 2002). By using single skeletal muscle fibers we have reduced diffusion distance enabling more efficient molecular substitution, which otherwise would have been exceedingly difficult to achieve in larger preparations.

Although the slow skeletal and cardiac TnC isoforms are identical, and our data demonstrates that cardiac TnC, when in fast-twitch muscle, can be oxidised and causes Ca\(^{2+}\)-sensitivity contractile dysfunction, in contrast Ca\(^{2+}\)-sensitivity did not change in permeablized slow-twitch muscle following GSNO treatment (Chapter 3. Fig C.5 and Table C.3). These data suggest that the endogenous anti-oxidants
associated with the native slow-twitch muscle myofibril may have a greater capacity to quench excess NO or remove modifications. Whether this also occurs in the cardiac muscle remains an open question, however, studies in cardiac tissue following ischemia-reperfusion injury have found modification to TnI and TnT (Labugger et al., 2000, McDonough et al., 1999) suggesting that endogenous reductants can be overwhelmed by oxidative stressors. Direct proteomic analysis of TnC at C84 in intact cardiomyocytes exposed to NO will assist in establishing whether this residue is responsible for NO mediated cardiomyocyte dysfunction.
General Discussion
Discussion

Oxidative stress is often associated with muscle dysfunction, but it is important to recognize not all oxidizing agents have the same impact. Specific oxidants may modify selective proteins or lipids, leading to a different functional outcome. Hydrogen peroxide and NO are two common oxidants with very different impacts and substrates. For example, preventing NO generation by the application of NOS inhibitors to whole muscle preparations results in an increase in force output for a given level of electrical stimulation (Kobzik et al., 1994a). In contrast, the addition of NO donors including SNAC, SNAP, GSNO and diethylamine-NO, consistently decrease the force-frequency relationship (Kobzik et al., 1994a). Data indicate that, independent of blood flow, low levels of NO cause a reduction in muscle fiber force output. In addition, in skinned, fast-twitch muscle fibers the NO donors, GSNO and SNAP reduced Ca\(^{2+}\)-sensitivity without affecting maximum contraction (Table C.1).

Furthermore, data presented herein, are consistent with published data (Andrade et al., 1998a, Posterino et al., 2003), reporting that H\(_2\)O\(_2\) applied to the fast-twitch muscle fiber during submaximal contraction, increases Ca\(^{2+}\)-sensitivity. Interestingly, following H\(_2\)O\(_2\)-mediated oxidative stress, Ca\(^{2+}\)-sensitivity can also be further increased by the exogenous application of the reductant, glutathione (Table C.1). These results suggest that in vivo, ROS specifically H\(_2\)O\(_2\), at low levels may be beneficial.
Both RNS and ROS target free sulphydryl groups on proteins, including proteins associated with the contractile apparatus. It had been suggested that NO and ROS may interact at similar sites on proteins and lipids and through competition, weak oxidants may confer protection from more damaging potent oxidizing agents (Reid, 1998). However, when we examined the functional effects of sequential addition of GSNO and H$_2$O$_2$ they retain their distinct effects, indicating that both molecules act independently leading to a net shift in Ca$^{2+}$-sensitivity (Fig C.4).

Interestingly the consequences of GSNO and H$_2$O$_2$ administration were different in fast- and slow-twitch fiber types. Fast-twitch fibers showed a small increase in Ca$^{2+}$-sensitivity when exposed to H$_2$O$_2$ and a decrease in Ca$^{2+}$-sensitivity when exposed to GSNO. However, slow-twitch fibers showed no changes in Ca$^{2+}$-sensitivity or maximum force when subject to the same protocols (Fig C.5). In a physiological setting, slow-twitch fibers are also more resistant to fatigue than fast-twitch fibers (Stephenson et al., 1998). Considering that protein isoform expression and metabolism define the two fiber types, two possible explanations may account for these differences including that; 1) slow-twitch fibers contain more endogenous anti-oxidants, including proteins which contain free thiols that may act as a buffer; 2) differences in reactivity of myofilament proteins” to oxidizing agents.

However, using a mutant C84S TnC that is not subject to sulphydryl oxidation at position cys84 enabled one to identify that the C84 TnC is the functional substrate for GSNO-mediated Ca$^{2+}$-desensitization (Fig D.6). The recombinant TnC used in substitution experiments was the cardiac/slow twitch isoform. When incorporated into
the fast-twitch fiber, sensitivity to GSNO was observed, indicating that other elements associated with the slow-twitch muscle myofilament were responsible for its insensitivity to GSNO. These results provide evidence that troponin isoform expression is not responsible for slow-twitch fiber insensitivity to GSNO leaving one to consider more seriously a role for endogenous myofilament associated antioxidants in slow-twitch fibers as a key mechanism providing protection against oxidative stress. Future studies will test this directly by substitution of skeletal muscle TnC into slow-twitch muscle fibers, followed by exposure to a NO donor.

Slow-twitch fibers rely primarily on oxidative phosphorylation to generate ATP and as a result generate a large amount of free radicals. So-called glycolytic fast-twitch fibers rely on glycolysis and to a lesser extent oxidative phosphorylation to generate ATP. To counter the higher production of oxidative molecules it seems plausible that slow-twitch fibers may have evolved with a greater complement of endogenous antioxidants associated with the myofilament. Even with the high concentration of NO donor and H₂O₂ used, slow-twitch fibers were able to overcome the oxidative insult. The presence of myofilament associated antioxidants, such as non-function protein free thiols, may also explain the slow-twitch fibers insensitivity to other oxidative molecules (Plant et al., 2001) and the “fatigue resistance” of slow-twitch muscle fibers (Stephenson et al., 1998). The results support the concept of endogenous anti-oxidants such as GSH or catalase being directly associated with the myofilament. An alternative hypothesis would be that slow-twitch muscle has a reserve of non-functional sulfhydryls which act as a sink to buffer oxidation.
The work of Van Eyk’s laboratory has demonstrated that cardiomyocyte derived TnI and TnT, lost to the blood serum following a MI or IRI, undergo a shift in isoelectric point (Labugger et al., 2002, Labugger et al., 2000, McDonough et al., 1999, McDonough et al., 2001). This suggests that the troponin complex is subject to oxidative modification following ischemia. However, our TnC substitution experiments wherein the reactive cysteine 84 residue of TnC was replaced with the less reactive serine, demonstrated that TnC was the functional substrate of GSNO mediated decline in Ca\(^{2+}\)-sensitivity (Fig D.6). Two-dimensional electrophoresis of GSNO treated skeletal muscle (Fig D.3) demonstrated that TnC is not the only myofilament protein modified during GSNO exposure. Myosin light chains have been suggested to be a possible substrate for GSNO, in fact we confirmed they were indeed modified. However, that C84S TnC substituted fibers were insensitive to GSNO despite this and other proteins in the filament being oxidized, indicates that in fast-twitch fibers, the functional substrate for GSNO leading to myofilament dysfunction is C84 TnC.

Moreover the fact that we used cardiac/slow isoform of recombinant TnC in these experiments may provide grounds for an extension of implications of this work to include possible outcomes in cardiac tissue. Slow-twitch muscle was insensitive to GSNO, likely due to a high endogenous anti-oxidant content preventing oxidation. However, many studies have provided evidence of oxidative damage in cardiac tissue following myocardial infarct (Labugger et al., 2000, McDonough et al., 1999, McDonough et al., 2001). The fact that it has been demonstrated that TnI oxidation follows MI, indicates that some oxidation is occurring and that cardiac muscle does not have the same anti-oxidant reserve as we observe in slow-twitch skeletal muscle.
This leads one to speculate that, following myocardial infarction C84 TnC oxidation may also be the substrate responsible for myocardial contractile dysfunction.

Although the specific nature of the modification of TnC was not directly assessed in this thesis, the GSNO mediated change in C84 TnC is likely due to a nitrosylation of the cysteine residue, as the GSNO mediated decline in Ca^{2+}-sensitivity was also seen when exposed to other NO donors such as SNAP and both are reversed with the addition of DTT (Hess et al., 2005).

**Limitations and future directions**

The skinned muscle fiber model allows for the isolation of the muscle fiber and the exclusion of the ion channels and soluble signaling proteins involved in EC-coupling. Although this study focuses on the identification of the myofilament basis for functional changes, other elements, found in intact muscle fiber models including ion channels, proteins involved in SR Ca^{2+} regulation, membrane potential and neuronal input are also likely to contribute to the NO mediated decline in muscle function.

Although we have identified C84 TnC as the functional substrate for GSNO mediated muscle fiber Ca^{2+}-desensitization the study could be improved through the use of authentic NO, DET NONOate, and nitrates or nitrites (Lundberg et al., 2008, Trottier et al., 1998). Nitric oxide donors have the potential to give spurious results as a bi-product of muscle fiber reaction with substrates produced during NO liberation. Although limited by the use of NO donors we reduced the possibility of spurious
results by confirming the results with two different exogenous NO sources, GSNO and SNAP, suggesting that NO mediates the changes in $\text{Ca}^{2+}$-sensitivity.

Evidence of TnI and TnT modification has been identified in MI, however, the specific modification of TnC was not investigated. We have shown both biochemical and functional evidence of TnC modification following NO exposure. Further studies are aimed to identify whether TnC is modified during pathologies such as IRI, coronary ligation and chronic limb ischemia in both skeletal muscle and cardiac muscle models. Protein isolation followed by mass-spectrometry of native TnC following IRI would confirm: 1) if TnC is post-translationally modified; 2) the specific target residue and 3) the form of modification present.

Calcium-binding to TnC is known to cause a series of conformational changes relayed through the troponin complex, altering protein binding affinities, specifically the binding affinity of tropomyosin to troponin. The decrease in tropomyosin-troponin binding affinity causes strong actin-myosin binding. Post-translational modification of TnC may result in the modification of protein binding affinities within the tropomyosin-troponin complex, specifically oxidation of Cys84 TnC may increase the tropomyosin-troponin binding affinity. These possibilities could be investigated using isolated proteins coupled with isothermal titration calorimetry.
Physiological sources of NO

There are three endogenous sources of NO; the $\text{Ca}^{2+}$-calmodulin dependent eNOS; nNOS; and $\text{Ca}^{2+}$-independent iNOS. To demonstrate the clinical relevance of NO production and muscle dysfunction, future studies will aim to identify the source of harmful, endogenous NO. During IRI several NOS isoforms are believed to be active during various stages. Briefly: 1) eNOS activity is increased in response to the ischemic event to vasodilate vessels and improve blood flow; 2) following reperfusion activated neutrophils release NO at several orders of magnitude greater via iNOS as a component of the inflammatory response. Nitric oxide production may be prevented by NOS blockers, such as L-NAME, however, they are non-specific, leading to blockade of all NOS isoforms. Nitric oxide synthase blocker administration has the potential to identify temporal importance of NO production during IRI through administration prior to, during and following ischemia. Nitric oxide synthase knockout mice of both the iNOS and eNOS isoforms have been developed and provide a specificity not seen in NOS blockade. However, the essential nature of eNOS and iNOS can lead to adaption of knockout mice through increased production of NO via the remaining NOS isoforms. The most effective knockout mouse model will be one that employs an inducible knockout, allowing for normal physiological function of NOS isoforms until the targeted blockade of iNOS, or eNOS expression is required.

Determining the extent to which endogenous NO contributes to muscle dysfunction in pathologies such as IRI and coronary occlusion is complicated by the formation of the vasoconstrictor, peroxynitrite from NO and $\text{O}_2^{-}$•. The abundance of the important
NOS co-factor BH4 is reduced as physical activity declines (Sindler et al., 2009) commonly associated with age, and can become limited during ischemia leading to $O_2^{•−}$ generation by NOS rather than NO. However, future spin paramagnetic resonance studies would do well to consider the formation of peroxynitrite rather than NO itself as the potentially most harmful product during IRI. Peroxynitrite generation may be limited by reducing the abundance of $O_2^{•−}$ present through the addition of SOD or, a membrane permeant superoxide dismutase mimetic, TEMPOL. However, concentrations of SOD or TEMPOL must be able to effectively compete with the favorable reaction of NO and $O_2^{•−}$. Prevention of peroxynitrite formation may improve vasodilatation during ischemia through increased bioavailability of NO and reducing the abundance of a vasoconstrictive RNS. Managing the balance between ROS and NO formation, presents a formidable challenge in the management of IRI (Matthews et al., 1996, Motohashi and Yamamoto, 2004, Reid, 1998).

**Significance**

The identification that slow-twitch muscle is relatively insensitive to GSNO and $H_2O_2$ may be of therapeutic value. Specifically, exercise and activity are important tools in the management of health. However, poor cardiac and vascular function may impair the ability to tolerate anaerobic exercise, mediated by fast-twitch skeletal muscle, due to oxidative stress further impairing muscle function. The fact that slow-twitch is insensitive to oxidative stress suggests the potential for a successful return to activity should employ slow-twitch muscle. By limiting exercise to slow-twitch muscle groups, the decline in $Ca^{2+}$-sensitivity, leading to contractile dysfunction in fast-twitch muscle, resulting from an increased ROS and RNS, may be reduced.
Nitrosylation is increasingly being recognized as an important component of cellular signaling likened to phosphorylation. Like phosphorylation, mediated by the balance of kinase and phosphatase activities, effective nitrosylation mediated signaling is dependent on maintaining appropriate redox status. Maintaining the redox balance in the face of an increased generation of NO, such as during IRI, will ultimately require the identification of novel therapies. As the addition of exogenous anti-oxidants currently remains equivocal (Bjelakovic et al., 2004, Miller et al., 2005, Vivekananthan et al., 2003) a novel target may be to enhance the production of endogenous anti-oxidants.
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