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THE ROLE OF ANTIOXIDANTS IN CARDIAC AND SKELETAL MUSCLE DURING CONDITIONS OF ENERGY DEFICIT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
The Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Paul F. Klawitter, M.D.

*****

The Ohio State University
2002

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ABSTRACT

ROS have been shown to play an important role in regulating the contractile state of both skeletal and cardiac muscle, in both normal and stressed conditions. Despite general acceptance that ROS play this role, the mechanisms by which they exert their effects is still unclear. This dissertation explores the mechanism by which ROS regulate contractile function in both skeletal and cardiac muscle exposed to conditions in which energy supply does not match energy demand.

A perfused rat heart model of ischemia and reperfusion, in which energy demand is unchanged but energy supply is diminished, was used to create this mismatch in cardiac muscle. An *in vitro* model of muscle fatigue, in which energy demand is increased out of proportion to energy supply, was used to create this mismatch in skeletal muscle.

I tested the hypotheses that during cardiac ischemia and reperfusion ROS: 1) inhibit metabolism and 2) participate in the degradation of troponin I (Tn I), two factors linked to the development of post-ischemic dysfunction. It is shown that ROS generated during ischemia promote post-ischemic energetic recovery during reperfusion through increases in contractile efficiency. It is also shown that ROS affect the pattern of troponin I degradation.

A novel model of global ischemia, which includes a period of low flow ischemia prior to reperfusion, was also developed. The addition of the low flow period more accurately reflects the clinical situation of cardiac arrest and may alter the generation of ROS as well as the energetic and functional recovery compared to standard models. It is
shown that a threshold level of flow exists below which energetic recovery is inhibited compared to prolonged complete ischemia.

To create an imbalance between energy supply and demand in skeletal muscle a new model of \textit{in vitro} fatigue was developed and verified. This novel model matches the tension time index (TTI) between groups. The data suggest that TTI is a primary determinant of \textit{in vitro} fatigue, regardless of frequency of stimulation and that ROS may not play a role in the development of fatigue.
Dedicated to my wife, Kristen,

and my children,

Brenna and Jack
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PUBLICATIONS

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FIELDS OF STUDY

Major Field: Biophysics
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CHAPTER 1

INTRODUCTION

The past ten years have witnessed an explosion in research regarding reactive oxygen species (ROS). What were once thought to be just harmful byproducts of cellular processes are now known to be important regulators of many cell functions (54, 67, 102, 175, 249). What may be most interesting is the finding that ROS, especially when present at low levels, are not only mediators of cell pathology but are also mediators of normal cell physiology (175, 192).

A variety of stimuli lead to ROS generation in both cardiac and skeletal muscle, including heat stress (251), hypoxia (62, 171), fatigue (190), ischemia (19) and reperfusion (252, 253). Contractile function in both skeletal and cardiac muscle may be altered by exposure to ROS (133, 31, 192). The mechanisms by which ROS lead to changes in contraction are not well known.

In this dissertation, I explore the role of oxidant-antioxidant balance in the regulation of muscle contraction. Both cardiac and skeletal muscles are examined in an attempt to uncover parallels between the two muscle types. A model of ischemia and reperfusion was used to study cardiac muscle and a model of fatigue was chosen to study
skeletal muscle. Both of these stimuli are known to result in contractile dysfunction that is attenuated by antioxidant therapy (28, 30, 70, 190, 191). Furthermore, both of these stimuli represent conditions of imbalance between energy supply and demand.

**Ischemia and Reperfusion**

As the name suggests, ischemia reperfusion (IR) injury refers to the effects of a period of ischemia and subsequent reperfusion on tissues or organs. All cell types and organ systems are at risk of being exposed to IR. Research across organ systems has revealed several ubiquitous findings. Among the most striking are that: 1) in the face of a relatively short ischemic time, the majority of the histologic injury appears to occur during the reperfusion period (117, 181), and 2) the generation of reactive oxygen species (ROS) and increases in intracellular Ca$^{2+}$ are key factors in the observed pathophysiology (27, 181).

Given the widespread nature of cardiovascular disease in society, IR in the myocardium is of particular interest. One aspect of myocardial IR injury that has received a great deal of attention is post-ischemic contractile dysfunction. When the period of ischemia is of short enough duration (generally, less than 20 minutes (27, 118)) there is no irreversible cellular injury and the contractile dysfunction is transient. This special case, in which there is normal blood flow but abnormal ventricular function that is eventually reversible, has been termed "myocardial stunning" (34).

The standard model of stunning involves a single episode of complete regional ischemia induced by occlusion of a single coronary vessel (26). However, similar phenomena occur in several situations of decreased blood flow, such as global ischemia, as well as situations of increased O$_2$ demand, such as exercise in the face of a coronary
stenosis (26). The degree to which the pathogenesis of these different entities overlaps is currently unknown (27). The portion of this dissertation that deals with ischemia and reperfusion will be directed at elucidating the role of ROS in the myocardial stunning that follows global ischemia.

Global ischemia, as opposed to regional ischemia, refers to the situation in which there is limited or no blood flow to the entire heart. The most common clinical situation in which this occurs is cardiac arrest. Cardiac arrest refers to the condition in which the mechanical pumping function of the heart ceases. This is most commonly a result of the development of ventricular fibrillation (VF)(8), a dysrhythmia characterized by chaotic electrical activity (98) that becomes uncoupled from contraction.

Survival rates for cardiac arrest are dismal. The overall rate (survival to discharge from the hospital) ranges from 0-20% depending on locale (18). Unfortunately, rates of 20%, such as reported for King County, Washington (151), are outliers. Results similar to the Chicago rate of 1.6% (17) are more typical. Interestingly, a much larger proportion of people suffering from VF have a return of spontaneous circulation (ROSC) following VF, but only about 20% of this group survives to discharge (38). The majority of deaths occur within 48 hours secondary to neurologic or cardiovascular dysfunction. A better understanding of the pathophysiology of ischemia and reperfusion in the heart has the potential to make a significant impact on a devastating disease.

As was stated earlier, Ca\textsuperscript{2+} overload and generation of ROS appear to be the chief mediators of IR injury in most organ systems studies. The myocardium is no exception, and the pathogenesis of myocardial stunning appears to be linked to both Ca\textsuperscript{2+} overload and generation of ROS.
The Role of $\text{Ca}^{2+}$ as a Mediator of Stunning

The role of $\text{Ca}^{2+}$ overload in the pathogenesis of myocardial stunning is well accepted. Direct measurements of [$\text{Ca}^{2+}$], have demonstrated increases during both ischemia and reperfusion (41, 158) of as much as 1-3 $\mu$M. Even in the absence of ischemia, $\text{Ca}^{2+}$ overload is sufficient to reproduce the functional derangements seen in stunned myocardium (135). Inhibition of $\text{Ca}^{2+}$ overload using low $\text{Ca}^{2+}$ perfusion (12) attenuates contractile dysfunction.

Both extracellular and intracellular sources may contribute to increases in free intracellular $\text{Ca}^{2+}$. The majority of the $\text{Ca}^{2+}$ responsible for the increases seen during ischemia and reperfusion is thought to arise extracellularly and enter via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCE) (101, 223).

During ischemia, a fall in pH leads to extrusion of $\text{H}^+$ through the $\text{Na}^+/\text{H}^+$ exchanger (NHE), with a concomitant rise in $\text{Na}^+$. Simultaneously, a fall in ATP decreases the rate at which the $\text{Na}^+/\text{K}^+$ ATPase can extrude $\text{Na}^+$. This leads to an increase in [$\text{Na}^+$], (as reviewed in (128)). The excess $\text{Na}^+$ is extruded through the NCE in exchange for $\text{Ca}^{2+}$. It is thought that this is further aggravated upon reperfusion, when realkalinization activates the NHE and increases the flux of $\text{Na}^+$ into the cell (as reviewed in (128)).

Evidence for the role of these ion channels in the pathophysiology of myocardial stunning is provided by studies in which channel function was inhibited. Inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger by reperfusion with acidotic (136) or high $\text{Na}^+$ (144) perfusate attenuates post-ischemic dysfunction. Selective inhibition of the NHE decreased $\text{Ca}^{2+}$ overload and improved postischemic function (215).
Intracellular Sources of Ca\(^{2+}\)

There are two main intracellular reservoirs of Ca\(^{2+}\) that may contribute to Ca\(^{3+}\) overload: the sarcoplasmic reticulum (SR) and the mitochondria. As discussed in more detail later, the SR is the organelle responsible for releasing and sequestering the Ca\(^{2+}\) responsible for driving contraction. It has been shown that ROS promote Ca\(^{2+}\) release from (96, 129, 140) and inhibit sequestration (126, 161) into the SR. This could result in an increase in \([\text{Ca}^{2+}]_i\) and provides a link between ROS generation and Ca\(^{3+}\) overload in the development of stunning.

A second possible source of Ca\(^{2+}\) is the mitochondrion. Mitochondria sequester and release Ca\(^{2+}\) in order to regulate metabolism, respiration and \([\text{Ca}^{2+}]_i\) (as reviewed in (148, 209)). Several groups have shown that the mitochondria increase the sequestration of Ca\(^{2+}\) during ischemia and then release it during reperfusion (113, 170). Others have provided evidence that the SR, and not the mitochondria, is the significant subcellular source of Ca\(^{2+}\) during ischemia and reperfusion (239). While the mitochondria represent a possible source for increases in \([\text{Ca}^{2+}]_i\), their role as a source of Ca\(^{2+}\) in ischemia and reperfusion is not clear.

Mechanisms of Stunning

The specific mechanism by which the increase in Ca\(^{2+}\) leads to stunning is not yet known. Current research is focused on the activation of calcium-dependent proteases, enzymes that when activated by Ca\(^{2+}\) degrade other proteins. The calpains make up one family of calcium-dependent proteases. The specific calpain that has received the most attention is calpain I. The contractile consequences of myocardial stunning can be
reproduced with calpain I incubation (88). Furthermore, calpain I activity is increased after ischemia-reperfusion in isolated hearts (250), and inhibition of the protease attenuates contractile dysfunction (162, 230).

**Reactive Oxygen Species as a Mediator of Myocardial Stunning**

**Reactive Oxygen Species**

Free radicals are molecular species with one or more unpaired electrons in a molecular orbit. In describing a radical species, it may be referred to as an "n"-centered radical, where n is the atomic species having the unpaired electron(s). For example, superoxide (O$_2^-$) is an oxygen-centered radical. Free radicals are usually unstable and capable of rapidly reacting with other molecules. (Interestingly, O$_2$ is also a radical species with two unpaired electrons. Their orbital configuration acts to stabilize the molecule.) Oxygen centered radicals are a part of a larger family of reactive oxygen species, which may or may not be radicals. For example, H$_2$O$_2$ is a ROS but not a radical.

**Superoxide**

Superoxide is created from the one electron reduction of O$_2$ as a byproduct of normal cellular function (83, 191). It may be produced by a number of mechanisms, though the two sources that have received the most attention in the realm of ischemia and reperfusion are the mitochondria and xanthine oxidase.

The main function of mitochondria is to generate energy in the form of ATP. This process requires the transfer of electrons through a series of oxido-reductases,
ending with the four-electron reduction of $O_2$ to $2H_2O$. It is commonly stated that 1-2% of $O_2$ consumption may be attributed to superoxide generation in the mitochondria through an "unintended" one electron reduction of $O_2$ (33).

Xanthine oxidase is a key enzyme in the catabolism of purine nucleotides, including adenosine, the nucleotide associated with ATP. Normal enzyme function results in the production of superoxide, as well as other ROS (discussed later) (141). The xanthine oxidase reaction is commonly taken advantage of in the laboratory to generate exogenous ROS.

Additional, non-mitochondrial, membrane bound oxidases also generate superoxide in the normal course of their function. The best known of these is the NADPH oxidase present on the membrane of phagocytic cells, which generate superoxide as a means of defense (as reviewed in (11)). It has recently been demonstrated that functional components of the NADPH oxidase are present on non-phagocytic cells (154).

Additional superoxide producing enzymes include lipoxygenase (16) and cyclooxygenase (199). Both enzymes are active in the metabolism of arachidonic acid. This may point to a possible link between $Ca^{2+}$ overload and ROS as arachidonic acid is hydrolyzed from a phospholipid by phospholipase A$_2$ (PLA$_2$), a calcium activated lipase.

**Hydrogen Peroxide ($H_2O_2$)**

As was mentioned before, hydrogen peroxide is a non-radical ROS. The majority of $H_2O_2$ is generated through the dismutation of two molecules of superoxide into one molecule of $H_2O$ and one molecule of $H_2O_2$ (reaction 1).

$$2H^+ + 2 O_2^- \xrightarrow{\text{SOD}} H_2O_2 + H_2O$$  (reaction 1)
This reaction is catalyzed by the enzyme superoxide dismutase (SOD) (discussed later). \( \text{H}_2\text{O}_2 \) may also be formed directly through the xanthine oxidase reaction (141) and in peroxisomes, an intracellular organelle in which \( \text{H}_2\text{O}_2 \) is used in the neutralization of toxic molecules and the oxidation of fatty acids. \( \text{H}_2\text{O}_2 \) is relatively non-reactive and plays a role as a signaling agent (175). A second role is the formation of additional ROS, such as hypochlorous acid (\( \text{HOCI} \)) and hydroxyl radical, through secondary reactions.

**Hydroxyl Radical (\(^{\cdot}\text{OH}\))**

Hydroxyl radical is formed through the Fenton reaction (reaction 2).

\[
\text{H}_2\text{O}_2 + \text{Cu}^{2+}/\text{Fe}^{2+} \rightarrow ^{\cdot}\text{OH} + \text{OH}^- + \text{Cu}^{2+}/\text{Fe}^{3+}
\]

(reaction 2)

The transition metals may be reduced by superoxide:

\[
\text{Cu}^{2+}/\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Cu}^{+}/\text{Fe}^{2+} + \text{O}_2
\]

(reaction 3)

The sum of reactions 2 and 3 form the Haber-Weiss reaction and illustrate the importance of transition metals in the formation of \(^{\cdot}\text{OH}\). This recycling reaction is extremely important since it may produce large quantities of \(^{\cdot}\text{OH}\), which is one of, if not the, most reactive oxygen-centered radicals and a chief mediator of cell injury (as reviewed in (37)).

**Endogenous Antioxidants**

The cell has developed an elaborate system of enzymatic and non-enzymatic defenses to protect itself from excess levels of ROS. In 1969 McCord and Fridovich (164) described superoxide dismutase (SOD), the first known enzymatic antioxidant.
SOD catalyzes reaction 1 at diffusion limited rates. There are two SOD isoenzymes in eukaryotes, a Mn-SOD (242) present in mitochondria, and a Cu/Zn-SOD (164) present in the cytosol. There is also a distinct extracellular Cu/Zn-SOD (159).

Catalase catalyzes the dismutation of \( \text{H}_2\text{O}_2 \) into oxygen and \( \text{H}_2\text{O} \). It is located mainly in the peroxisome and functions to detoxify different substrates such as phenols and alcohols. Thioredoxin peroxidase is also capable of directly reducing peroxides, such as \( \text{H}_2\text{O}_2 \) and alkyl hydroperoxides (42, 43).

Glutathione peroxidase (GPx) is yet another enzyme capable of reducing \( \text{H}_2\text{O}_2 \), using reduced glutathione (GSH) as a substrate. It is also capable of reducing other peroxides to alcohols (reaction 4).

\[
\text{ROOH} + 2\ \text{GSH} \rightleftharpoons \text{ROH} + \text{GSSG} + \text{H}_2\text{O}
\] 

(Reaction 4)

Aside from acting as a substrate for GPx, GSH may also act as a non-enzymatic antioxidant through direct interaction with oxidants to yield oxidized glutathione (GSSG). GSSG is reduced to GSH by the NADPH-dependent glutathione reductase.

Vitamin E and vitamin C are also non-enzymatic antioxidants. Vitamin E is a lipid soluble vitamin found in cell membranes. It can react with lipid peroxyl radicals and prevent a chain reaction of lipid peroxidation from occurring in the cell membrane. It is then reduced by vitamin C back to its active form (212). Vitamin C can also directly reduce superoxide and other peroxides (37). Vitamin C is recycled back to its reduced form by both glutathione (163) and thioredoxin (168).

Under normal circumstances, the endogenous AOX systems present in cells are able to maintain the ROS at almost undetectable levels. However, under certain
conditions of increased ROS generation the endogenous AOXs may be overwhelmed. Such conditions are frequently taken advantage of in the laboratory to study ROS. One method of study involves the addition of exogenous AOXs to augment the endogenous AOX capacity. By comparing tissues or cells that had exogenous AOXs added to those that did not, one can gain indirect evidence of the effects of ROS. In addition, adding exogenous AOX with different mechanisms of action potentially provides additional information regarding the specific species involved. This is the method used in this dissertation.

**ROS in Ischemia and Reperfusion**

In addition to ROS generation as a result of normal cell physiology, a number of stresses, such as hypoxia (62, 171), heat (251) and toxin exposure (216) result in an increase in ROS production. In one of the models of cell stress investigated in this dissertation, myocardial ischemia/reperfusion, the production of additional ROS are considered critical in the development of post-ischemic contractile dysfunction (220).

There is both direct and indirect evidence in support of a reactive oxygen-mediated mechanism of myocardial stunning. Various antioxidants (AOX), including SOD and catalase (173), hydroxyl radical scavengers (207) and iron chelators (207), have been shown to attenuate myocardial stunning. EPR spectroscopy has been used to show a burst of ROS upon reoxygenation (207, 253). The time course of the generation varies between groups but appears to peak within the first few minutes of reperfusion. This burst can be attenuated by the use of AOXs, which simultaneously attenuate
stunning (207). Finally, there is a direct correlation between the duration of ischemia, a prime determinant of the extent of injury, and the amount of ROS generated upon reperfusion (30).

There is also mounting evidence that ROS are generated during ischemia (19, 235, 130). In some ways, the generation of excess ROS during a period of decreased O$_2$ seems paradoxical. However, as O$_2$ levels decrease the cell develops a condition of "reductive stress," a situation in which excess reducing equivalents, such as NADH accumulate. As will be discussed later, this condition can promote generation of radicals.

The phenomenon of ROS generated during ischemia has received less attention than the burst of radicals seen upon reperfusion. Their significance, if any, is unclear. Their potential importance as cell signals is pointed out by recent studies in the field of cardioprotection, the condition in which brief periods of ischemia protect the myocardium from subsequent, longer ischemic insults (as reviewed in(40)). It has been seen that attenuation of the ROS generated during the brief ischemic period prevents the development of the cardioprotective phenotype (233, 46, 229).

**Sources of ROS in Ischemia and Reperfusion**

Any of the mechanisms discussed above could potentially account for the production of ROS during ischemia and reperfusion. The majority of attention has been focused on the mitochondria. Studies examining ROS generated during both ischemia (234) and reperfusion (179, 180) have implicated the mitochondrion as the chief source of ROS. Vanden hoek et al. (234) were able to inhibit the appearance of ROS by blocking the anion channel responsible for superoxide's movement across the mitochondrial membrane.
Work done in the laboratory of Hans Nohl points to a possible mechanism leading to generation by ROS in addition to the basal production (178). In conditions of high reductive stress, mimicking the situation seen during ischemia, it was seen that ubiquinone, one of the mobile electron carriers in the mitochondrial electron transport chain, moved from the interior to the exterior of the membrane (178). This allowed ubiquinone to facilitate the one electron reduction of O₂, resulting in the production of superoxide (178). This offers an explanation for the paradoxical generation of ROS during low O₂ states.

More recently, it was shown that any alteration in mitochondrial membrane fluidity was sufficient to increase ROS generation, through alterations in the mobility of portions of the electron transport chain (93). The conditions present during ischemia and reperfusion, namely increased NADH, have been correlated with changes in membrane fluidity (178).

The conditions present during ischemia and reperfusion also suggest alternative sources of ROS. The adenylate kinase reaction generates one ATP and one AMP from two ADPs. As ATP levels fall in the myocyte, and ADP levels rise, the adenylate kinase reaction leads not only to increased ATP but also AMP. AMP may then be degraded, resulting in increased substrate for the xanthine oxidase reaction, as discussed earlier. Thompson-Gorman and Zweier (227) have shown that this reaction was a significant source of ROS during ischemia and reperfusion. As expected, the amount of ROS generated was proportional to the fall in ATP.
Mechanisms of ROS-Induced Contractile Dysfunction

Despite the plethora of evidence linking ROS to contractile dysfunction following ischemia and reperfusion in the myocardium, the mechanism by which this occurs is poorly understood. Two potential targets known to be susceptible to ROS that may contribute to post-ischemic dysfunction are cellular metabolism and the contractile protein apparatus.

Excitation-Contraction Coupling

In order to understand how either oxidative modification of metabolism or contractile proteins may lead to contractile dysfunction it is first necessary to have a basic understanding of the mechanism of contraction in the myocyte. The process by which force is developed is termed excitation-contraction coupling. As the name implies, it is generally thought of as a two-step process (for detailed discussion see (23)).

When a myocyte is at rest there exists a potential difference across the membrane of approximately -90 mV (negative on the inside). This potential difference is maintained by the active (energy consuming) pumping of ions, chiefly K⁺ and Na⁺, across the membrane. When there is a local depolarization to approximately -40 mV, voltage-gated Na⁺ channels in the vicinity open and allow Na⁺ to travel passively down its electrochemical gradient into the cytosol. This causes further depolarization distant from the initial site and a wave of depolarization is begun. The depolarization travels along the cell membrane and eventually down invaginations, known as "T tubules."

Within the T tubules are voltage-sensitive Ca²⁺ channels. Within the cytosol, and adjacent to the T tubules, is the sarcoplasmic reticulum (SR) which is the site of Ca²⁺ sequestration. Ryanodine receptors (so named because they bind the substance
ryanodine) are located upon the SR membrane and face the Ca\(^{2+}\) channels on the T tubule. When the T tubules are depolarized, Ca\(^{2+}\) enters the cytosol through the voltage-gated Ca\(^{2+}\) channels and binds to the ryanodine receptors. The binding of Ca\(^{2+}\) to the ryanodine receptors results in the release of Ca\(^{2+}\) from the SR. This Ca\(^{2+}\) may bind to additional ryanodine receptors, resulting in further Ca\(^{2+}\) release in a process known as calcium induced calcium release (39, 48). (In skeletal muscle, the voltage sensitive receptor on the t-tubule may actually interact directly with the ryanodine receptor during depolarization to induce Ca\(^{2+}\) release (as reviewed in (147).) The increase in cytosolic [Ca\(^{2+}\)] is the signal that allows contraction.

The contractile apparatus is composed of parallel thick (myosin polymers) and thin (actin polymers) filaments (as reviewed in (189)). Myosin is made of thick filaments, which run in parallel to actin polymers. A lever arm, composed of myosin heavy chain, spans the gap between actin and myosin. There are two myosin light chains attached to the end of the heavy chain, forming a globular head that can interact with actin.

Actin monomers polymerize into double helical filaments. Upon the actin filament is the tropomyosin molecule, which is an α helix in contact with seven actin monomers, as well as adjacent tropomyosin molecules. Attached to each tropomyosin complex is a troponin regulatory complex. Troponin has three subunits: Troponin C (Tn C), which binds Ca\(^{2+}\), Troponin I (Tn I), the inhibitory subunit, and Troponin T (Tn T), which binds tropomyosin.

Myosin heavy chain is an ATPase (hydrolyzes ATP to provide energy for an action). The hydrolysis of ATP causes a structural change in the myosin arm, which acts
to "cock" it for later contraction (217, 238). The ADP and Pi resulting from the ATP hydrolysis remain bound to the myosin. Myosin, when bound to ADP and Pi, has a strong affinity to bind to actin, but troponin and tropomyosin block this.

When a membrane depolarization results in Ca$^{2+}$ release, as described above, Ca$^{2+}$ binds to Tn C. This causes Tn I to release actin, allows tropomyosin to move into the groove in actin and releases the inhibition for actomyosin binding (61, 152). The myosin head now attaches to actin in a weakly bound conformation and undergoes a transition to a strongly bound form (189). Myosin is now able to release Pi and ADP, which causes the lever arm to move back into the "uncocked" state (124, 189). This is the power stroke, which results in myosin sliding past actin, the foundation of contraction. ATP can now bind to myosin, which leads to a decrease in myosin's affinity for actin and causes the myosin head to release (156). This returns the contractile proteins back to the initial state of the cycle, as described here. As long as Ca$^{2+}$ is present and bound to Tn C, the cycle will continue. When Ca$^{2+}$ is sequestered back into the SR, or extruded from the cell, cycling is stopped and the muscle will relax.

Anything that directly or indirectly interferes with excitation-contraction coupling would lead to contractile dysfunction. In this dissertation, two of many possible targets of ROS modification which may contribute to a disruption in normal excitation-contraction coupling are investigated: metabolism and contractile proteins.

**Metabolism**

**Role of Metabolism in Ventricular Dysfunction**

Metabolism refers to the processes by which the cell generates ATP. The maintenance of metabolism, and especially glycolysis, during ischemia and reperfusion is
clearly linked to maintenance of ventricular function. The degree of functional recovery following ischemia is directly related to the degree of glycolytic activity (58). Multiple groups have shown that continued glycolytic flux during reperfusion plays a key role in maintaining post-ischemic function (119, 120). Cross et al. (59) demonstrated that continued glycolytic activity preserved Na\(^+\) homeostasis and attenuated postischemic dysfunction. Decreases in glycolytic flux during ischemia are associated with early development of contracture (138), the increase in end diastolic pressures that is a hallmark of myocardial stunning following I/R.

Since ATP provides the energy that enables contraction, it would seem intuitive that maintenance of ATP supply would be associated with maintenance of contractile function. However, it is not clear that energetic status is a key determinant of contractile function at the energy status typical of ischemic myocardium.

Several studies support a relationship between cardiac function and a variety of energetic parameters including 1) phosphorylation potential (52), 2) PCr levels (142), and 3) the ratio of PCr/ATP (127). Other groups have evidence against such a relationship (109, 205, 219). Hoerter et al. (109) showed that despite depletion of PCr to 15% of normal and ATP to levels undetectable by NMR, normoxic hearts were still able to develop 65% of their normal systolic pressure and had no change in diastolic pressure. Saupe et al. (205) monitored energetics while decreasing perfusion pressures slowly, and showed that the decrease in myocardial contractility preceded any change in cardiac energetics. The role of energetic status in post-ischemic ventricular dysfunction is not yet clear.
An alternative explanation for the improved recovery of hearts with increased metabolic, and especially glycolytic, rates is an improvement in ion homeostasis. Glycolysis produces ATP that is preferentially used to maintain ion gradients across membranes (59, 74, 138, 155). It is especially important during periods of Ca\(^{2+}\) overload (2, 120), such as is seen during IR. As was discussed earlier, Ca\(^{2+}\) overload may be a consequence of the inability to maintain Na\(^{+}\) homeostasis. Therefore, the role of glycolysis in maintaining Ca\(^{2+}\) homeostasis may be, in part, an indirect consequence of improved Na\(^{+}\) homeostasis (59). Additionally, inhibition of glycolytic flux leads to an accumulation of sugar-phosphates, which can interfere with calcium homeostasis, independent of ATP changes (145).

**ROS Regulation of Metabolism**

In addition to the evidence linking metabolism to improvements in post-ischemic ventricular function, there is also evidence that metabolism may be regulated by ROS. This is especially true of glycolysis, a key component of metabolism responsible for maintaining contractile function (as discussed above). Nemoto et al. (175) demonstrated that ROS generated by the mitochondria shift glucose metabolism away from glycolysis through a complex cell-signaling cascade. It has also been shown that larger concentrations of ROS may directly inhibit the enzymes in the glycolytic pathway (57, 115, 123).

Lastly, there is indirect evidence that ROS inhibit metabolic recovery during ischemia and that this may contribute to post-ischemic contractile function. Previous studies in a model of prolonged cardiac arrest demonstrated that antioxidant (AOX) treatment, either before (248) or after (5) ischemia, promoted high-energy phosphate
recovery during reperfusion and improved post-ischemic ventricular function. However, in these studies the hearts were subjected to thirty minutes of ischemia, a duration that probably resulted in necrosis (13, 27). It is not known if the mechanisms that lead to dysfunction after infarction are the same as those in myocardial stunning. Therefore, it is not yet clear that inhibition of metabolism is the means by which ROS lead to ventricular dysfunction after shorter periods of ischemia.

**Contractile Proteins**

Currently, a large amount of research effort is being directed at the study of contractile protein modification as the possible ultimate lesion responsible for myocardial stunning. This has been prompted by several findings in the last decade.

One of the initial discoveries pointing to a connection between stunning and protein modification was the finding that stunned myocardium typically demonstrates not a decrease in Ca$^{2+}$ availability, but a decrease in Ca$^{2+}$ sensitivity (84, 110). That is, a given [Ca$^{2+}$] results in less force production in stunned vs. normal myocardium. Furthermore, there is a decrease in maximal force generation at saturating [Ca$^{2+}$] (84).

This has not been a universal finding and several groups have been unable to demonstrate any change in Ca$^{2+}$ sensitivity (71, 232). It has been suggested that this discrepancy may be explained by differences in sample preparation technique (91). For example, in the study by Geyer et al. (91) it was demonstrated that prolonged skinning of fibers, with complete removal of all soluble factors, resulted in fibers with no change in Ca$^{2+}$ sensitivity. On the other hand, “incomplete” skinning resulted in a decrease in Ca$^{2+}$ sensitivity, leading the authors to conclude that a soluble factor present after ischemia leads to the decrease in sensitivity.

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In any case, the consensus opinion is that stunned myocardium displays a decrease in Ca^{2+} sensitivity. This opinion led to investigation of potential culprits, namely modification of contractile proteins. As was mentioned above, the troponin complex appears to be the Ca^{2+} sensor, and investigation of this regulatory complex revealed a degradation of troponin I in a perfused rat heart model of myocardial stunning (85).

This finding has been repeated in different models (167, 232), though it is noticeably absent in others (134). However, the stunned phenotype can be reproduced in mutants expressing the fragmented troponin I (Tnl) (172), illustrating that this lesion is sufficient, if not absolutely necessary, to result in myocardial stunning. Furthermore, replacement of native Tn I in non-stunned muscle with Tn I taken from stunned myocytes results in the development of the stunned phenotype (165). None of these studies have demonstrated any changes in tropomyosin or troponin C or T in the absence of infarction (85).

**Troponin I**

As mentioned briefly above, Tnl is one of the subunits comprising the troponin regulatory complex, Tn C and TnT being the others. There are three isoforms of Tnl, one each for fast and slow twitch skeletal muscle and one for cardiac muscle (68, 186). Skeletal muscle Tnl is made of 181 amino acid residues, while cardiac Tnl has an additional 30-amino-acid peptide joined to its N-terminal (186). There is currently no crystal structure for Tnl available.

Purified Tnl has been shown to inhibit the actomyosin ATPase in a Ca^{2+}-dependent manner (99). Two regions of Tnl, residues 96-116 (218) and 128-148(228),
bind actin and are able to inhibit the actomyosin ATPase even when cleaved from the TnI molecule. The binding of TnI to actin and its ATPase inhibition are potentiated by tropomyosin (218).

Troponin I acts independently from tropomyosin by preventing the myosin heads from forming the weak interaction with actin (153). It acts in conjunction with tropomyosin by keeping tropomyosin out of the actin groove, which inhibits the transition of the weak and strong binding of myosin (153). When Ca\(^{2+}\) binds to troponin C, Tn I moves away from actin and towards troponin C and removes the steric blockage between actin and myosin (68, 213). Furthermore, this motion allows tropomyosin to move into the actin groove, at which point it no longer interferes with any interaction between myosin and actin (153). At this point contraction can occur as described above.

**Mechanisms of Troponin I Degradation**

If Tn I degradation was the ultimate lesion responsible for myocardial stunning following ischemia and reperfusion, one would expect it to be linked to Ca\(^{2+}\) overload and/or ROS, the two main events considered essential for myocardial stunning. There is evidence linking the proteolysis to Ca\(^{2+}\) overload. Activation of the Ca\(^{2+}\) dependent protease, calpain I, reproduces Tn I degradation as well as contractile dysfunction (85). In addition, reperfusion with low Ca\(^{2+}\) perfusate, which minimizes [Ca\(^{2+}\)], prevents degradation and attenuates stunning (85).

**ROS and Protein Degradation**

ROS may oxidize or reduce proteins. These alterations can mark the protein for degradation (63, 174). In typical circumstances, one of the main systems for marking and degrading altered proteins is the ubiquitin system. Ubiquitin is a 76-amino-acid protein
found in all eukaryotic cells. Ubiquitin is conjugated to the target protein in an ATP-dependent and enzyme-facilitated fashion (47). Multiple ubiquitins are conjugated to each other forming a branched chain (45). This chain acts as a signal for degradation by the 26 S proteasome (32, 197) (proteasome refers to a class of large, multicatalytic proteases (75)). However, following oxidant exposure, ubiquitin conjugation is inhibited (210), and the ubiquitin-ATP dependent pathway plays only a minor role in proteolysis (64, 80).

ROS may promote protein degradation in several ways. A variety of common proteases, including trypsin, pepsin and calpain, the Ca\textsuperscript{2+}-dependent protease already implicated in Tn I degradation, degrade oxidized proteins more rapidly than native proteins (66, 114, 196).

A unique pathway for degradation of oxidized proteins has also recently been described. The degradation is carried out by a large multicatalytic complex, containing both sulfhydryl- and serine-proteases (184). This proteasome is distinct from the complex involved in the ubiquitin-mediated degradation. An increase in exposure of hydrophobic residues in the oxidized protein appears to be the signal for degradation (94, 184).

ROS species have been shown to decrease Ca\textsuperscript{2+} sensitivity (87), though the mechanism is unclear. ROS may lead to Tn I degradation by multiple mechanisms. These include an increase in calpain activity (188), a mechanism that could link both Ca\textsuperscript{2+} overload and ROS generation. In addition, there is evidence of oxidant-mediated degradation of key proteins following ischemia and reperfusion in the brain (183).
Despite these compelling facts, there are no studies to date attempting to link Tn I degradation to ROS generation following ischemia and reperfusion in the myocardium.

**Myocardial Models of Ischemia and Reperfusion**

The phenomenon of ischemia and reperfusion can take many forms in the myocardium. All ischemia and reperfusion can be broken into one of two categories depending on whether the entire heart (global ischemia) or a portion of the heart (regional ischemia) is subjected to ischemia, as would be seen during cardiac arrest or myocardial infarction, respectively. Most research done in the area of myocardial stunning has been in the area of regional ischemia (220). In models of regional ischemia, some device is surgically placed around a coronary artery, and ischemia and reperfusion are induced by manually opening and closing the device.

It is not yet clear if the pathophysiology of regional ischemia can be directly extrapolated to global ischemia (220). This dissertation is concerned with the role of ROS in myocardial stunning following global ischemia. In models of global ischemia, such as those used in this study, a Langendorf preparation is frequently employed. In this preparation the heart is removed intact from the animal's chest and a canulla is placed into the aorta. The heart is then perfused through the aorta at either constant pressure or constant flow. Stopping flow into the aorta induces global ischemia. Frequently, flow is completely stopped for some period of time and this is followed by immediate restoration of flow at original perfusion pressures (85). Whether this model applies to any kind of clinical situation is debatable. For example, if flow were started again during resuscitation it would not occur at original perfusion pressures. Current resuscitation
guidelines call for closed chest compressions, in an effort to provide blood flow to vital organs (1) prior to return of spontaneous circulation. Therefore models of cardiac arrest that don’t have an intervening period of low flow ischemia mimicking chest compressions between the complete ischemic period and reperfusion may not adequately reflect the clinical situation they are trying to model.

CPR is performed in the hope that the low flow generated will provide enough O$_2$ to extend organ viability during the period of arrest. With respect to the heart, it is hoped that CPR generated coronary flow will attenuate myocardial stunning, and thereby improve perfusion to all organ systems during the post-resuscitation period. However, the effect that this period of low flow has on the heart is unclear.

It seems intuitive that any decrease in total ischemic time would translate into improved post-ischemic function. However, it has been suggested that low levels of flow may actually be detrimental compared to continuing complete ischemia through decreasing intracellular pH and metabolite washout (137). Although this has been well studied in the brain and found not to occur in that system (137), the effects on the myocardium are less well understood. It has been shown that, in the myocardium, hypoxia results in more contractile dysfunction than ischemia, probably through washout of metabolites (169, 202). As discussed above, washout of H$^+$ from the extracellular space may lead to worsening Ca$^{2+}$ overload as the Na$^+$/H$^+$ exchanger (NHE) increases [Na$^+$]$_i$ in an attempt to resolve intracellular acidosis. Sufficiently low levels of flow may mimic the hypoxic situation and cause worsening post-ischemic function.

Although Takeo et al. (221) demonstrated improved post-ischemic function with reduced flow rates following global ischemia vs. immediate return of spontaneous

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circulation, they studied flow rates that were greater than those generated by standard CPR. Development of a model that included a period of low flow, prior to reperfusion, at levels typically seen during CPR would allow more accurate study of this important clinical condition. It would also help answer the question of whether or not CPR should even be performed prior to interventions that would immediately result in ROSC, such as defibrillation or bypass.

Muscle Fatigue as a Model of Contractile Regulation by ROS

Within this dissertation, a second model of contractile regulation by ROS was also considered. In contrast to the other work done, this model concentrated on fatigued skeletal muscle. This allowed exploration of potentially conserved mechanisms of regulation between the two muscle types. In addition, the stress that the myocardium and the skeletal muscle were exposed to was similar at a fundamental level. Both stresses involved situations in which the energy supply was not sufficient to meet demand. In the ischemic myocardium, energy supply fell out of proportion to demand. In the fatiguing skeletal muscle, energy demand rose out of proportion to supply. An additional model of hypoxia and reoxygenation in skeletal muscle paralleling the myocardial ischemia/reperfusion model was also employed. However, I was one of several people involved in that work and thus it was not included in this dissertation.

ROS have been shown to be present and necessary for maximum tension generation during non-fatiguing contractions (192). However, ROS may play an even more prominent role in the development of skeletal muscle fatigue during intense contraction (6, 9, 69, 70, 191, 193).
Skeletal muscle fatigue, and the mechanisms leading to it, have been the object of investigation for decades, if not centuries. Likewise, there have been many proposed definitions of fatigue. For the purpose of this dissertation, I will consider fatigue to be the inability of a given muscle to achieve a target tension development for a given condition. That is, if a rested muscle can achieve a tension "A" with contraction, after a series of repetitive or prolonged contraction the muscle may only be able to generate some tension less than "A". This muscle may then be considered fatigued. Fatigue may also be described as it relates to some specific condition. For example, the tension development at a particular frequency of stimulation (contraction from a series of consecutive depolarizations at some set frequency, for example 20 Hz), versus tension developed from a twitch (contraction from a single depolarization of the cell membrane).

**Mechanisms of Fatigue**

There are most likely multiple complex cellular mechanisms responsible for fatigue. In a classic paper on fatigue, Eberstein and Sandow (76) proposed that fatigue in their system (prolonged, low frequency stimulation) was secondary to disruption of excitation-contraction coupling. They proposed that the lesion responsible for fatigue was a failure of Ca\textsuperscript{2+} release from the SR. This was based on the finding that the application of caffeine, known to stimulate Ca\textsuperscript{2+} release from the SR, resulted in significant recovery of force in fatigued muscle. This finding has been reproduced in several labs (97, 243, 247), and Westerblad and Allen (243) have demonstrated a decline in [Ca\textsuperscript{2+}], directly.

The mechanism responsible for the alteration in Ca\textsuperscript{2+} cycling is not entirely clear, though it appears to involve changes in bioenergetics. Indirect evidence includes the
observation that declines in Ca\textsuperscript{2+} release occur later in fibers with higher oxidative capacity (231) and that it occurs more rapidly when muscle is fatigued in anaerobic conditions (149).

Furthermore, Allen et al. (3) were able to use caged ATP, released with a Xenon flash, to increase [ATP] \textsubscript{i} in a step fashion in fatigued muscle. This led to an increase in Ca\textsuperscript{2+} release and an increase in tension development completely accounted for by the increase in available ATP. Although, as is discussed later, gross [ATP] is essentially unchanged during typical fatigue, the authors theorized that a microenvironment within the cell, in the area of the SR release channel, actually has an ATP deficit. They hypothesized that the caged ATP, which is freely diffusible, was able to replenish the supply in this critical site (244).

Changes in Ca\textsuperscript{2+} cycling are not solely responsible for fatigue. The process of excitation-contraction coupling is complex with many additional or complementary sites of potential inhibition. There are two cellular events that have classically been purported to result in fatigue, and have been linked to disruption in EC coupling. These are the decrease in intracellular pH as a result of lactic acid production secondary to glycolysis and ATP hydrolysis, and the increase in inorganic phosphate (Pi) secondary to ATP hydrolysis by the myosin ATPase (56).

In a classic study, Cooke et al. (56) gave convincing evidence that H\textsuperscript+ and Pi inhibit muscle contraction. They demonstrated that Pi inhibits contraction not through a decrease in the free energy of ATP hydrolysis (amount of energy available to be used for work when ATP is hydrolyzed to Pi and ADP), but rather through inhibition of Pi release.
from the actomyosin complex. As described above, the release of Pi is thought to initiate the powerstroke. The causes of inhibition secondary to low pH were less clear and it was hypothesized to be a result of effects on protein structure.

Godt and Nosek (95) took these ideas further and applied them to hypoxic cardiac and fatigued skeletal muscle. The intracellular milieu in fatiguing skeletal muscle and hypoxic cardiac muscle are very similar (125). In their paper, Godt and Nosek (95) found similar decreases in Ca\(^{2+}\) sensitivity and maximal force in both skeletal and cardiac muscle by mimicking the intracellular milieu seen during hypoxia/fatigue. This presents the intriguing possibility that the decrease in force production seen in hypoxic skeletal muscle is due to the same mechanisms that lead to depression in force following fatiguing stimulation.

Recently it has been suggested that pH may not play as critical a role in fatigue development at body temperature. Force production has been shown to recover more rapidly than intracellular pH (200). Both Westerblad et al. (245) and Pate et al. (185) have shown that changes in force production and velocity of contraction secondary to low pH are only manifest at low temperature, and that this inhibition is gone at physiologic temperatures.

A limitation in energy supply has classically been proposed as an additional mechanism of fatigue. Aside from the possible association it may have with changes in Ca\(^{2+}\) release, as described above, it would seem intuitive that declines in energy availability would be linked to declines in contractile force. It would also seem intuitive that since hydrolysis of ATP provides the energy for contraction one would expect to see declines in [ATP] associated with fatiguing stimulation. However, it has been
demonstrated repeatedly that ATP remains at baseline levels well after fatigue has set in (237), and it is only a late finding, at a time of almost complete contractile failure, that [ATP] declines (237).

This may be explained by the presence of phosphocreatine in muscle cells. Phosphocreatine (PCr) is used as both an energy sink and shuttle in select tissue, notably muscle and nerve (201). The reaction (Reaction 5) in which PCr is hydrolyzed, and ADP phosphorylated to ATP is catalyzed by the enzyme creatine kinase (CK).

\[
{\text{PCr + ADP}} \rightleftharpoons \text{CK} \rightarrow {\text{ATP + Creatine}}
\]

Reaction 5

The equilibrium is heavily shifted to the right, so that ATP is almost immediately resynthesized.

Therefore, one would expect to see a fall in PCr with fatiguing stimulation, and indeed, it has been shown that after 10 seconds of maximal exercise, power output decreases and is correlated with a significant fall in PCr (108). Additionally, muscle force is related to muscle PCr during fatigue as well as recovery (108). It may be argued that contractile function is related to PCr through the generation of Pi associated with its hydrolysis. However, it has been shown that creatine supplementation, which led to increased PCr at rest, as well as increased Pi during stimulation, actually decreases fatigue (105). These findings suggest that limitations in energy supply play a significant role in the development of fatigue during high-intensity exercise.

**Frequency-Dependent Fatigue**

The above discussion touches on just some of the many possible mechanisms, working alone or in concert, that may be responsible for the development of muscle
fatigue. Although the details of the cellular events that lead to fatigue are not yet entirely clear, what has become apparent is that the mechanisms of fatigue, and recovery from fatigue, may depend on the frequency of stimulation.

High Frequency Fatigue

Several decades ago it became clear that the rate of fatigue development was dependent upon stimulation frequency, and occurred relatively rapidly when frequencies greater than 50 Hz were used (24). Investigation of this "high frequency fatigue" led to several consistent observations. Reducing the frequency of stimulation after the onset of fatigue rapidly reverses the loss in force (122). Decreasing extracellular [Na⁺] or increasing extracellular [K⁺] can exacerbate the loss of force (25). Finally, the muscle action potential is both slowed and decreased in amplitude during fatigue suggesting involvement of membrane ion homeostasis (25).

These features are consistent with an increase in extracellular [K⁺] during high frequency fatigue that affects transmission of the action potential along the membrane (121). It has been hypothesized that these changes are even greater in the t-tubule, because the closed space would multiply the effect of any ion imbalance. Westerblad et al. (247) were able to show that Ca²⁺ gradients were altered secondary to failure of action potential propagation, confirming that this mechanism is possible.

Low Frequency Fatigue

Low frequency fatigue is a more complex entity. Classically, low frequency fatigue is characterized by a group of findings that occur after any fatiguing activity, not necessarily after low-frequency stimulation. That is, it generally refers to the contractile
response to low stimulation frequencies after fatigue. However, it is common for people to refer to fatigue after low frequency stimulation as "low frequency" fatigue.

There are several characteristics of classic low frequency fatigue, and the name was coined after it was observed that fatiguing stimulation at any frequency most severely depressed force generation at low frequencies (77). That is, consider a muscle that has just been subjected to a fatiguing protocol. It is then stimulated at 20 Hz and 100 Hz. If the tensions generated are expressed as a fraction of the tension generated by the respective frequencies at rest, the 20 Hz stimulation would generate less tension than the 100 Hz stimulation.

In addition, this fatigue persists for hours or days in the absence of metabolic or electric disturbance (78). This is in contrast to high frequency fatigue, which can resolve in seconds to minutes, and displays profound disturbances in the action potential (121). These observations led to the hypothesis that low frequency fatigue is a result of damage to muscle fiber structure or uncoupling of excitation-contraction.

Either a decrease in Ca²⁺ sensitivity or a decrease in Ca²⁺ release could explain alterations in EC coupling. Westerblad et al. (246) showed that for a given frequency there was reduced [Ca²⁺], but no change in the relationship between [Ca²⁺] and tension. This indicates that the aspect of low frequency fatigue that involves EC coupling is reduced SR Ca²⁺ release.

Role of ROS in Fatigue

As was mentioned earlier, the mechanisms and characteristics of muscle fatigue have been the object of study for decades, if not centuries. However, only recently has there been awareness that ROS may play a role in the development of fatigue. It has been
shown that muscle generates ROS at rest, and that this production increases with stimulation (191). It has also been demonstrated that AOX treatment decreases tension generated at low frequencies in rested muscle, indicating that ROS may be necessary for optimal function of unfatigued muscle (192).

The situation in fatigued muscle seems quite different. Several groups have shown that AOX treatment attenuates fatigue (6, 9, 69, 70, 191, 193). However, the mechanism by which AOX confer this "protection" is as yet unknown. Elucidation of this mechanism(s) is one of the goals of this dissertation. As discussed in detail above ROS can inhibit metabolism and high-energy phosphate production. Limitation of high-energy phosphate supply has been implicated as a factor responsible for fatigue (discussed above). Therefore, protection of metabolism by AOX treatment as a mechanism for its fatigue-sparing effects is an attractive hypothesis.

Before this possibility can be addressed, some factors that are associated with AOX treatment during ischemia must be addressed. As was mentioned above, AOX treatment preferentially depresses tension generated at low frequencies by rested muscle (192). This means that if two muscles, one AOX-treated and one untreated, are being compared at the same frequency of stimulation, the AOX-treated muscle will generate less initial force. If it is then stimulated but exhibits less fatigue, it is unclear if the sparing is secondary to the AOX or just that the tissue was being stimulated to produce less force at the beginning. In an effort to overcome this limitation Khawli et al. (133) altered the initial frequency of stimulation to match initial tension generation between groups. However, as has been pointed out, fatigue may be frequency-dependent and this "solution" may not be valid.
The second issue, and the one that bears most on studies of metabolism in fatigue, has to do with a concept referred to as the tension-time index (TTI). TTI can be defined as the average force developed by a rhythmically contracting muscle, expressed as a fraction of baseline maximum tetanic tension. Therefore, TTI increases directly with duty cycle (time of contraction / total contraction-relaxation time) and the average force generated during contraction. It is roughly analogous to the work performed by shortening contractions. The origins of the tension-time index concept arose from the observation that a primary determinant of myocardial O₂ consumption in the heart is the product of heart rate times the accumulated area under the systolic pressure curve (204).

The TTI is believed to be a key determinant of the development of skeletal muscle fatigue (20). Its application to the study of human skeletal muscle fatigue, in a wide variety of muscle fiber types, has shown that voluntary contractions with TTI values 0.15 or more ultimately result in muscle fatigue (20, 206). At TTI above 0.15, the level of fatigue was directly related to increases in TTI (20). The metabolic cost of isometric contractions is also directly proportional to TTI (60).

The role of TTI in the study of AOX and fatigue must be considered. Any intervention that attenuates fatigue, by definition, will result in greater tensions being generated by the treated muscle compared to the untreated by the end of the stimulation protocol. This will result in a greater TTI for the treated tissue, since its average pressure will now be greater. This means that this tissue will have had greater metabolic costs over the course of the protocol and will have been subjected to a more fatiguing stimulus. This will confound any comparison of either fatigue or energetic availability that is made.
Therefore, development of a model of fatigue that matches metabolic costs between groups would be invaluable for addressing the question of whether or not AOX attenuate fatigue through increased high-energy phosphate availability.

Summary

Understanding of the role of ROS in regulating cell physiology as well as pathophysiology has grown dramatically in the past decade. In this dissertation, I attempt to expand our understanding of the role of these important compounds. Specifically, I explore the role of oxidant-antioxidant balance in the regulation of muscle contraction. Two models are employed in which muscle contraction is thought to be regulated by ROS. The first is a model of myocardial ischemia and reperfusion; the second is a model of fatigue in skeletal muscle. These two models address the role of ROS produced secondary to a stress that is fundamentally the same. Both models create situations in which the supply of energy is not sufficient to meet the demands for energy. Each model achieves this imbalance in a unique way. In the myocardium, energy supply is depressed out of proportion to the depression in energy demand. In the skeletal muscle, the demand for energy is increased out of proportion to the increase in supply.

The overall theme of understanding the role of oxidant-antioxidant balance in the regulation of muscle contraction is addressed through the following aims:

1) To test the hypothesis that ROS generated during ischemia and/or reperfusion promote the loss of and prevent the recovery of high-energy phosphates.

Rationale: ROS species have been shown to inhibit various aspects of metabolism, such as glycolysis, that would potentially interfere with maintaince of high-
energy phosphates. Post-ischemic contractile dysfunction has been correlated to the level of glycolytic flux. By attenuating the oxidant stress generated during ischemia and reperfusion, through the addition of exogenous AOX, the effect of ROS on high-energy phosphates is indirectly determined.

2) To test the hypothesis that ROS generated during myocardial ischemia and/or reperfusion play a role in the degradation of Troponin I.

Rationale: Troponin I degradation may be the final affector of myocardial stunning. ROS species have been shown to promote protein degradation. By attenuating the oxidant stress generated during ischemia and reperfusion, through the addition of exogenous AOXs, the effect of ROS on Troponin I degradation is indirectly determined.

3) To develop a model of myocardial ischemia and reperfusion that includes a period of low flow perfusion, prior to full reperfusion, to better mimic the clinical condition of cardiac arrest with CPR.

Rationale: Current perfused heart models of cardiac arrest fail to include the period of low flow ischemia that typically occurs prior to return of spontaneous circulation. This period of intermediate perfusion and oxygen availability may have profound effects on the functional and energetic recovery of the hearts. Furthermore, the generation of ROS as a function of oxygen tension is complex and the intervening period of low oxygen tension following ischemia and prior to reperfusion may alter the overall
amount and effect of ROS. A model of global ischemia that includes a period of low flow ischemia is characterized in the anticipation that it will be used to address these questions in the future.

4) To develop a model of muscle fatigue in which the metabolic cost and contractile activity is controlled between groups.

Rationale: The presence of exogenous AOX attenuates the development of muscle fatigue. One possible mechanism by which they may exert their effects is through the maintainance of metabolism and an increase in high-energy phosphate availability. Current models of in vitro fatigue have several problems that would hinder study in this area. A model of fatigue that resolves these problems by matching TTI between groups is developed.

5) To test the hypothesis that AOX treatments attenuate muscle fatigue in a novel stimulation model in which metabolic costs and contractile activity are matched.

Rationale: The presence of exogenous AOX attenuates the development of muscle fatigue. A new model of in vitro muscle fatigue was developed that allows further exploration of the mechanisms involved. To verify it as a valid model, it was used to determine the effects of various AOX treatments on skeletal muscle fatigue. Results were compared to the effects using more common methods.
The findings associated with each aim will be presented in its own chapter. With the exception of aim 5, all aims have been submitted as original research papers and will be presented in typical journal article format.
CHAPTER 2

THE GOOD RADICAL: REACTIVE OXYGEN SPECIES GENERATED DURING ISCHEMIA ENABLE ENERGETIC RECOVERY DURING REPERFUSION

Introduction and Rationale

Return of spontaneous circulation following cardiac arrest is associated with post-ischemic ventricular dysfunction. This dysfunction may represent a form of myocardial stunning, the reversible inhibition of contractile function following a period of ischemia too brief to cause infarction (27). The burst of reactive oxygen species (ROS) seen upon reperfusion is considered one of the key factors in the development of myocardial stunning following regional ischemia (27). Lower levels of ROS are also generated during ischemia (19, 235, 130) and are considered an important factor in the development of cardiac preconditioning (13, 234). While the role of the ROS burst at the time of reperfusion is well studied, the role of ROS generated either during or following the global ischemia, as experienced with cardiac arrest, is less clear (27).

Previous studies found that in a model of prolonged cardiac arrest antioxidant (AOX) treatment, either before (248) or after (5) ischemia, promoted high-energy
phosphate recovery during reperfusion and improved post-ischemic ventricular function.

In these studies, the hearts were subjected to thirty minutes of ischemia, a duration that probably resulted in necrosis (13, 27). Whether or not such AOX treatment would influence energetic recovery or contractile function after shorter periods of ischemia that do not result in necrosis is unknown. It is likely that the pathophysiology leading to post-ischemic contractile dysfunction is not necessarily the same in stunned and infarcted myocardium (27).

A study was done on hypoxic skeletal muscle (51), analogous to global ischemia in the heart. It was found that treatment during hypoxia with N-acetylcysteine (NAC), which increases intracellular [glutathione] (226) and scavanges hydroxyl radical (10), or Tiron, a metal chelator and $\text{O}_2^{-}$ scavenger (100), during hypoxia preserved high-energy phosphates and improved contractile function. In the present study, we test the hypothesis that treatment with NAC or Tiron will preserve high-energy phosphates and improve post-ischemic contractile function in a model of global ischemia, simulating cardiac arrest, that likely results in stunning rather than infarction. In addition we attempt to distinguish the effects of ROS generated during reperfusion from those generated during ischemia by selectively attenuating oxidant stress in the ischemic period, compared to attenuation during the reperfusion period.

Methods

Preparation and Isolated Heart Perfusion

Male Sprague-Dawley rats weighing 350-450 g supplied by Harlan (Indianapolis, IN) were used in accordance with guidelines of the National Institutes of Health and the approval of the Ohio State University Institutional Laboratory Animal Care and Use
Committee. Twelve hours prior to the experiment, animals were fasted with free access to water. Rats were anesthetized using an intraperitoneal injection of sodium pentobarbital (50-65 mg/kg). The right superficial jugular vein was isolated and heparin (1000 U/kg) was administered. The trachea was cannulated and the rats were ventilated with room air using a rodent ventilator.

A midsternal thoracotomy was performed and a steel cannula (outside diameter of 3-4 mm) was placed through an aortotomy into the aorta and secured with a suture. While in situ, hearts were immediately perfused with Krebs-Henseleit bicarbonate buffer with the addition of 0.2 mM caprylic (octanoic) acid at a constant pressure of 85 mm Hg. Octanoic acid was added to better match the substrates available to the heart in vivo. Buffer was bubbled with 95/5% O₂/CO₂ to attain a pH of 7.4.

The heart was quickly excised from the chest and transferred to a Langendorf apparatus while continuously perfused. Perfusion pressure was maintained at 85 mm Hg with 37°C Krebs-Henseleit buffer. A water-filled latex balloon, attached to a pressure transducer by a stainless steel gavage needle, was inserted through the left atrium into the left ventricle for measurement of left ventricular (LV) pressure. The heart was submerged in a jacketed, temperature-controlled glass chamber and allowed to equilibrate for 15 min. The balloon volume was set to maintain a LV end diastolic pressure of 5 mm Hg. A heart performance analyzer (Digi-Med, Micro-Med, Louisville, KY) analyzed the signal from the pressure transducer. Developed pressure, ventricular end diastolic pressure (EDP) and rate pressure product (RPP; product of LV developed pressure and heart rate) were measured continuously.
An automated blood gas machine (Instrumentations Laboratory Critical Care Laboratory Synthesis 45) was used to measure PO$_2$ of the perfusate at the level of the aortic cannula and the effluent from the pulmonary artery. The difference between the two measurements was used to calculate oxygen consumption (MVO$_2$). The ratio of RPP to MVO$_2$ was used as a measure of contractile efficiency (143, 211).

**Perfusion Protocol**

Following the 15 minute equilibration period the hearts continued with a baseline period of 10 minutes of perfusion with oxygenated buffer. Flow was then stopped by turning a stopcock and the hearts were subjected to 20 minutes of warm ischemia. This was followed by 30 minutes of reperfusion at baseline perfusion pressures and PO$_2$. This protocol does not result in myocardial infarction as measured by lack of creatine kinase release during reperfusion (unpublished observation).

The control hearts received only oxygenated buffer throughout the protocol. The experimental groups were perfused according to protocol with buffer containing 10 mM Tiron (100), or 4 mM N-acetylcysteine (82, 10). Each experimental group was further divided into two groups. One group received AOX treatment during baseline perfusion only and therefore the AOX was already loaded into the tissue. The other experimental group received AOX during reperfusion only. A final group received a bolus infusion of 40 cc of 5 mM deferoxamine, an iron chelator, at the onset of ischemia. There were 5-8 hearts studied in all groups.

**Metabolite Analysis**

Additional experiments, separate from those done to determine functional results, were performed using an identical perfusion protocol. However, the hearts were

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freeze-clamped at the end of baseline, the end ischemia and the end of reperfusion and were freeze dried and stored at -80°C for later analysis. The hearts were ground under liquid nitrogen, metabolites extracted in 0.6 N perchloric acid and neutralized with NaHCO₃. Neutralized extracts were analyzed spectrophotometrically for lactate, PCr, ATP, Cr and Pi using standard methods (22, 214). N=6-8 for all groups.

Details for measurements are as follows:

**Lactate measurements**

Add 0.2 ml of neutralized perchloric acid extract and 0.2 ml of 40 mM NAD to 2.5 ml hydrazine/glycine buffer (0.4 M hydrazine; 0.5 M glycine; pH 9.0). Measure extinction coefficient (E₁) at 340 nm. Add 0.02 ml LDH (about 5 mg prot/ml). Mix. Allow to stand in water bath at 37°C for 30 minutes. Measure second extinction coefficient (E₂). Concentration of lactate in cuvette (mmol/l) is \( \frac{(E₂ - E₁)}{6.3 \times 10^3} \).

**Creatine Phosphate and ATP measurements**

Reagents:

0.2 M Tris/HCL, pH 7.8

MgCl₂: 40.6 mg in 2 ml H₂O

Glucose: 9 mg in 1 ml H₂O

NADP⁺: 10 mg in 1 ml H₂O

ADP: 20 mg in 3.5 ml H₂O. adjust pH to 7.0 with NaOH. Bring final volume to 4 ml.

50 mM Tris/HCl, pH 7.8

Glucose 6-phosphate dehydrogenase (G6PDH): 700 U/ml

Hexokinase (HK): 1400 U/ml
Creatine Kinase (CK): dissolve 10 mg CK powder (350 U/mg) in 0.3 ml 50 mM tris + 0.25 ml ADP solution.

Prepare reaction buffer by combining 10 ml 0.2 M Tris, 2 ml MgCl₂, 1 ml NADP⁺, 1 ml glucose and 15 μl G6PDH. Add 100 μl neutralized perchloric acid extract to 1 ml buffer.

Measure absorbance at 340 nM (E₁). Add 5 μl HK. Wait 8 minutes. Measure absorbance at 340 nM (E₂). Add 5 μl HK and measure absorbance at 340 nM (E₃). Extinction due to HK (EHK) is equal to E₃-E₂. Add 20 μl CK and wait 12 minutes.

Measure absorbance at 340 nM (E₄). Add 20 μl CK and wait 8 minutes. Measure absorbance at 340 nM (E₅). Extinction due to CK (ECK) is equal to E₅-E₄.

Concentration of ATP in cuvette (mmol/l) is \( \frac{(E₂-E₁-EHK)}{(6.3 \cdot 10^3)} \).

Concentration of PCr in cuvette (mmol/l) is \( \frac{(E₅-E₄-ECK)}{(6.3 \cdot 10^3)} \).

Creatine measurements

Reagents

0.2 M Glycine, pH 9.0

MgCl₂: 40.6 mg in 2 ml H₂O

Phosphoenolpyruvate: 10 mg in 0.5 ml H₂O

NADH : 5 mg in 1 ml H₂O

ATP : 4.5 mg in 0.5 ml H₂O.

KCl: 56 mg in 0.75 ml H₂O

Pyruvate Kinase (PK): 2000 U/ml

Lactate Dehydrogenase (LDH): 2750 U/ml

Creatine Kinase (CK): dissolve 20 mg CK powder (350 U/mg) in 0.4 ml Glycine buffer.
Prepare reaction buffer by combining 12 ml 0.2 M glycine, 1 ml MgCl₂, 0.5 ml NADH, 0.3 ml phosphoenolpyruvate, 0.5 ml ATP, 0.75 ml KCl, 50 μl LDH and 10 μl PK. Add 100 μl neutralized perchloric acid extract to 1 ml buffer. Measure absorbance at 340 nM (E₁). Add 25 μl CK. Wait 8 minutes. Measure absorbance at 340 nM (E₂). Add 25 μl HK and measure absorbance at 340 nM (E₃). Extinction due to CK (E₉) is equal to E₃−E₂. Concentration of creatine in cuvette (mmol/l) is (E₂−E₁−E₉)/(6.3×10⁳).

Inorganic Phosphate measurements

Prepare sample buffer by dissolving 1 g ascorbic acid and 0.25 mg ammonium molybdate in 20 ml H₂O each. Combine when dissolved. Add 1.42 ml pure H₂SO₄. If solution turns blue it is contaminated. Bring to final volume of 50 ml. Prepare Pi standards between 0.25 mM and 2.0 mM. Also, prepare a 5 mM ATP and 10 mM PCr standard.

Combine 1 ml buffer, 1 ml H₂O and 50 μl sample. Mix and wait for 75 seconds. Measure absorbance at 820 nM. Create a standard curve with Pi standards and use this to calculate Pi present in samples. To account for Pi in samples that resulted from breakdown of ATP and PCr during the procedure measure Pi present in ATP and PCr standards. This fraction, combined with the [ATP] and [PCr] measured in samples can be used to correct the measured Pi.

Data Analysis

High-energy phosphates were expressed as μmoles/ g dry wt. All values are expressed as means ± SEM. Significance of differences between means was determined by ANOVA, with Tukey’s or Dunnet’s post hoc tests performed, as applicable. Values of p <0.05 were considered statistically different.
Results

AOX Treatment prior to Ischemia

Functional Results: Treatment with Tiron depressed baseline LVF, and this was reflected in significantly lower developed pressure in the Tiron group compared to all other groups (Table 2.1). There were no other baseline differences between groups (Table 2.1). There was no difference in developed pressure or end diastolic pressure (EDP) at the end of reperfusion between any groups (Table 2.1).

However, there was a decrease in efficiency of contraction (143, 211) during reperfusion in the groups treated with NAC and deferoxamine (Fig. 2.1). There was a trend towards lower efficiency in the Tiron treated group, though this did not reach the level of statistical significance. None of the AOX treatments had an effect on baseline efficiency (data not shown).

High Energy Phosphates: Neither Tiron nor NAC had any effect on PCr or ATP during baseline perfusion (Fig. 2.2 and 3). The deferoxamine group was treated identically to the control group during baseline, and received the AOX as a bolus immediately prior to ischemia and therefore baseline energetic measurements were not performed.

Treatment with any of the AOXs prior to ischemia had no significant effect on ATP levels at the end of ischemia when compared to untreated controls (Fig. 2.3). In contrast, the PCr at the end of ischemia was almost twice as high in the Tiron treated hearts compared to either untreated, NAC or deferoxamine treated hearts (Fig. 2.2).

An increase in glycolytic flux during ischemia is one possible explanation for the increase in PCr seen in hearts treated with Tiron prior to ischemia. Lactate, the product
of anaerobic glycolysis, was quantified in the control and Tiron treated hearts as an indirect measure of glycolysis. There were no significant differences between groups (data not shown).

The effect of AOX treatment at the end of reperfusion on energetic status was markedly different than that measured at the end of ischemia. Treatment with any of the AOXs prior to ischemia inhibited recovery of ATP during reperfusion compared to control (Fig. 2.3). In addition, pretreatment with either Tiron or deferoxamine led to decreased levels of PCr at the end of reperfusion compared to control (Fig. 2.2).

**Treatment with AOX post-Ischemia**

A second set of experiments was performed in which hearts received either Tiron or NAC only during reperfusion. Neither AOX improved functional recovery during reperfusion (Table 2.2). Unlike treatment with AOXs prior to ischemia, hearts that received AOXs during reperfusion showed no change in either ATP or PCr at the end of reperfusion (Fig. 2.2 and 3).

**Treatment with deferoxamine prior to prolonged ischemia**

Because we were unable to show improvement in post-ischemic contractile function with deferoxamine, as Williams et al. (248) have, we attempted to more closely replicate their study by duplicating their prolonged ischemic time in an additional group of animals. Similar to the results seen in the groups subjected to a shorter ischemia time, treatment with deferoxamine prior to ischemia resulted in no improvement in post-ischemic function compared to control hearts when both groups were subjected to 30 minutes of ischemia (data not shown).
Discussion

In this study, none of the AOXs given either before or after ischemia attenuated post-ischemic contractile dysfunction. When given prior to ischemia, Tiron increased the PCr available to the myocardium only during ischemia but not during reperfusion. All of the AOXs studied, when given prior to ischemia, prevented bioenergetic recovery during reperfusion. The uniformity of the results in all AOX groups treated prior to ischemia suggests an oxidant-mediated mechanism and not a drug effect. AOX treatment given only during reperfusion had no effect on bioenergetics.

Functional Results

The lack of functional improvement with AOX treatment was unexpected. After initial experiments with Tiron and NAC showed no functional improvement, additional experiments using a bolus of deferoxamine prior to 20 or 30 minutes of ischemia were performed. This protocol was previously shown to result in improved functional and bioenergetic recovery in perfused rabbit hearts (5). Similar to the Tiron and NAC results, this provided no attenuation of contractile dysfunction in our model, leading to the conclusion that the functional response to AOXs is not ubiquitous and may be animal species dependent.

It is currently accepted that, aside from the use of SOD as a sole agent, AOX treatment attenuates myocardial stunning (27). However, review of the literature reveals several other examples of the failure of AOXs to preserve post-ischemic contractile function (208, 225, 226). The buffer-perfused rat heart has also been shown to have a variable response to AOXs as protective agents against myocardial stunning (55, 222). Despite the presence of the characteristic ROS burst upon reperfusion (89), the
buffer-perfused, isolated heart may not be a good model for studying the effects of AOX on post-ischemic contractile dysfunction following global ischemia over this time frame.

Energetic Results

*Energetics at the End of Ischemia:* Treatment with Tiron prior to ischemia led to an increase in available PCr at the end of the ischemic period. The increase seen could be secondary to increased energy production. Glycolysis can be inhibited by ROS (57, 115, 123). If so, it would have been expected that lactate levels would increase with Tiron treatment by preserving glycolysis but this was not observed in our data. We therefore feel it is unlikely that the changes in energetics at the end of ischemia are secondary to increases in glycolysis, though a direct measurement of glycolytic flux has not been made.

An alternative explanation is that the hearts treated with Tiron consumed less energy during the ischemic period. For example, if ROS impaired the integrity of the cell membrane, more energy would be consumed maintaining ion gradients. If attenuation of the ROS by Tiron prevented this breach in membrane integrity, there would be less demand for energy consumption by active ion transport.

*Energetics at the End of Reperfusion:* Contrary to what was seen at the end of ischemia, all AOX treatments, when given prior to ischemia, showed an inhibition of high-energy phosphate recovery during reperfusion. There was no effect on energetic recovery during reperfusion when the AOX was given only during reperfusion.

This suggests that the low level of ROS generated during ischemia (19, 235), as opposed to the large burst seen at reperfusion (89), may play a signaling role that promotes energetic recovery during reperfusion. One possibility is that, during ischemia,
the low levels of ROS lead to an increased rate of energy production during reperfusion. For example, as mentioned, though high levels of ROS inhibit glycolysis, in some systems exposure to low levels of oxidants is known to stimulate glycolysis (92, 106, 157).

An alternative explanation is that ROS generated during ischemia led to a decreased energetic cost of contraction during reperfusion. This is supported by our data, which demonstrates a decreased efficiency of contraction during reperfusion in hearts treated with AOX. One possible explanation for changes in efficiency is suggested by work done in the area of cardiac preconditioning. It has been shown that ROS generated during an initial ischemic insult are necessary for the induction of the preconditioned state (13, 234). It is thought that the mechanism by which ROS stimulate preconditioning is through the activation of protein kinase C (PKC) (13, 53). It has also been demonstrated that activation of PKC increases contractile efficiency (177). It is possible that ROS generated during ischemia increase efficiency by some PKC-regulated mechanism during reperfusion and that this was blocked by the administration of AOX prior to the onset of ischemia.

The energetic results are in conflict with prior studies, which showed an improvement in post-ischemic energetic recovery when AOXs were given either prior to ischemia (248), or upon reperfusion (5). However, in these studies rabbits were used as opposed to rats, and an ischemia time of 30 minutes was used. This duration of ischemia is probably associated with myocardial necrosis (13, 27) and may be associated with different pathophysiologic mechanisms than myocardial stunning (27). Either of these factors may explain the apparently discrepant results.
Conclusions

In conclusion, we have shown that AOX treatment either prior to ischemia, or upon reperfusion, has no effect on post-ischemic contractile function in this model of global ischemia. Treatment with Tiron prior to ischemia increased the [PCr] at the end of ischemia. This was not through an oxidant-mediated inhibition of glycolysis. However, treatment with any of the AOXs prior to ischemia actually prevented energetic recovery during reperfusion. The data suggest that the mechanism by which ROS generated during ischemia promote energetic recovery is through an increase in contractile efficiency. In contrast, ROS generated during reperfusion did not appear to play a role in energetic regulation. These findings suggest a physiologic, rather than pathologic, role for ROS generated during ischemia.
### Table 2.1: Developed Pressure (Dev Pressure) and End Diastolic Pressure (EDP) measured at the end of reperfusion in hearts that received an AOX prior to ischemia. Mean ± SEM. *p<0.05, ANOVA

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Dev. Pressure (mmHg)</th>
<th>EDP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline Reperfusion</td>
<td>Baseline Reperfusion</td>
</tr>
<tr>
<td>Ctrl</td>
<td>11</td>
<td>110±7 56±6</td>
<td>5.4±0.4 27.4±4.6</td>
</tr>
<tr>
<td>Tiron</td>
<td>5</td>
<td>79±10* 38±10</td>
<td>5.3±0.6 41.6±6.8</td>
</tr>
<tr>
<td>NAC</td>
<td>10</td>
<td>118±6 64±6</td>
<td>5.7±0.4 34.9±4.2</td>
</tr>
<tr>
<td>Def</td>
<td>6</td>
<td>107±10 69±9</td>
<td>4.3±0.8 25.5±8.8</td>
</tr>
</tbody>
</table>

### Table 2.2: Developed Pressure (Dev. Pressure) and End Diastolic Pressure (EDP) measured at the end of reperfusion in hearts that received AOX at the start of reperfusion. Mean ± SEM.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Dev. Pressure (mmHg)</th>
<th>EDP (mmHg)</th>
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<tr>
<td></td>
<td></td>
<td>Baseline Reperfusion</td>
<td>Baseline Reperfusion</td>
</tr>
<tr>
<td>Ctrl</td>
<td>7</td>
<td>125±7 58±7</td>
<td>5.5±0.4 27±7</td>
</tr>
<tr>
<td>Tiron</td>
<td>8</td>
<td>131±10 45±7</td>
<td>5.7±0.6 43±6</td>
</tr>
<tr>
<td>NAC</td>
<td>11</td>
<td>134±5 56±6</td>
<td>5.1±0.8 38±5</td>
</tr>
</tbody>
</table>
Figure 2.1: Efficiency of contraction, estimated as the ratio of the rate-pressure-product (developed pressure • heart rate) to oxygen consumption, measured at the end of reperfusion. Treated groups received the designated AOX prior to onset of ischemia as described in Methods section. Error bars represent the SEM. ANOVA with Tukey's post hoc.
**Figure 2.2**: Creatine phosphate measured at the end of baseline, ischemia or reperfusion. The group designated as "Reperfusion A" received the indicated treatment prior to onset of ischemia only. The group designated as "Reperfusion B" received the indicated treatment during reperfusion only. Details are described in the methods section. Error bars represent the SEM. * p<0.01 compared to time matched untreated. ANOVA with Tukey's post hoc.
Figure 2.3: ATP measured at the end of baseline, ischemia or reperfusion. The group designated as "Reperfusion A" received the indicated treatment prior to onset of ischemia only. The group designated as "Reperfusion B" received the indicated treatment during reperfusion only. Details are described in the methods section. Error bars represent the SEM. * p < 0.01 compared to time matched untreated. ANOVA with Tukey's post hoc.
CHAPTER 3

THE ROLE OF REACTIVE OXYGEN SPECIES IN THE DEGRADATION OF TROPONIN I DURING ISCHEMIA AND REPERFUSION

Myocardial stunning is the reversible depression in ventricular function following an ischemic insult too brief to cause infarction (27). A rise in intracellular Ca\(^{2+}\) and a burst of reactive oxygen species upon reperfusion appear to be the key events initiating the cascade that leads to stunning (27). Both Ca\(^{2+}\) overload (146) and ROS (86) exposure have been shown to decrease myocardial Ca\(^{2+}\) sensitivity, which is thought to be the final effector of ventricular dysfunction (84, 110).

It has been suggested that the decline in Ca\(^{2+}\) sensitivity is secondary to troponin I degradation (85). A link between Ca\(^{2+}\) overload and troponin I degradation was suggested by the finding that reperfusion with low Ca\(^{2+}\) / low pH buffer prevented the troponin I proteolysis and attenuated the post-ischemic contractile dysfunction (85).

Although ROS have also been shown to decrease the Ca\(^{2+}\) sensitivity of myofilaments (87), their role in the breakdown of troponin I is not known. ROS are known to modify protein function (65, 174) and promote proteolysis (63, 64, 174). Antioxidants have been shown to attenuate oxidative modification of proteins in...
non-cardiac models of ischemia/reperfusion injury (183). In this study we test the hypothesis that troponin I degradation can be prevented through attenuation of ROS by various antioxidants. In addition, we attempt to distinguish the role of ROS generated during ischemia from those generated during reperfusion through selective attenuation during the ischemic period compared to attenuation during the reperfusion period.

Methods

Preparation and Isolated Heart Perfusion

Male Sprague-Dawley rats weighing 350-450 g obtained from Harlan (Indianapolis, IN) were used in accordance with guidelines of the National Institutes of Health and the approval of the Ohio State University Laboratory Animal Care and Use Committee. Twelve hours prior to the experiment, animals were fasted with free access to water. Rats were anesthetized using an intraperitoneal injection of sodium pentobarbital (50-65 mg/kg). The right superficial jugular vein was isolated and heparin (1000 U/kg) was administered. The trachea was cannulated and the rats were ventilated with room air using a rodent ventilator.

A midsternal thoracotomy was performed and a steel cannula (outside diameter of 3-4 mm) was placed through an aortotomy into the aorta and secured with a suture. While in situ, hearts were immediately perfused with Krebs-Henseleit bicarbonate buffer with the addition of 0.2 mM caprylic (octanoic) acid at a constant pressure of 85 mm Hg. Buffer was bubbled with 95/5% O2/CO2 to attain a pH of 7.4.

The heart was quickly excised from the chest and transferred to a Langendorf apparatus while continuously perfused. Perfusion pressure was maintained at 85 mm Hg with the same 37°C Krebs-Henseleit buffer. A water-filled latex balloon, attached to a
pressure transducer by a stainless steel gavage needle, was inserted through the left atrium into the left ventricle for measurement of left ventricular (LV) pressure. The heart was submerged in a jacketed, temperature-controlled glass chamber and allowed to equilibrate for 15 min. The balloon volume was set to maintain a LV end diastolic pressure of 5 mm Hg. A heart performance analyzer (Digi-Med, Micro-Med, Louisville, KY) analyzed the signal from the pressure transducer. Developed pressure during reperfusion for each heart was calculated as the fraction of baseline developed pressure (Reperfusion Dev. Pressure mm Hg / Baseline Dev. Pressure mm Hg).

**Perfusion Protocol**

Following the 15 minute equilibration period the hearts continued with a baseline period of 10 minutes of perfusion with oxygenated buffer. Flow was then stopped by turning a stopcock and the hearts were subjected to 20 minutes of warm (37°C) ischemia. This was followed by 30 minutes of reperfusion at baseline perfusion pressures and PO₂. This protocol does not result in myocardial infarction as measured by lack of creatine kinase release (unpublished observation).

The control hearts received only oxygenated buffer throughout the protocol. The experimental groups were perfused according to protocol with buffer containing 10 mM Tiron, a superoxide scavenger (100), or 4 mM N-acetylcysteine, a precursor of reduced glutathione (82) and a scavenger of hydroxyl radical (10). Each experimental group was further divided into two groups. One group received AOX treatment during baseline perfusion only, the other experimental group received AOX during reperfusion only. Hearts were freeze-clamped at the end of baseline, ischemia or reperfusion and kept frozen at −80°C for later analysis (n=5-8 for all groups).
Contractile Protein Analysis

Samples were analyzed utilizing gel electrophoresis and immunoblotting as previously described (194, 195). At the end of the perfusion protocol, left ventricular samples (25-40 mg), free of visible fat and connective tissue, were homogenized after adding 30 μl of sample buffer per mg tissue. Samples were heated to 65° C for 2 min and centrifuged for 5 min at 12,000 rpm in an Eppendorf centrifuge. The supernatant was diluted 1:10 with sample buffer, and 3-4 μL were loaded onto 12% acrylamide mini gels. Gels were run for 1.5 hrs and the proteins are subsequently transferred to nitrocellulose. Using anti-troponin I antibodies (anti-Tnl monoclonal antibody Mab 81-7 [Spectral Diagnostics, Philadelphia]), immunoblotting was done to visualize troponin degradation products. Bands recognized by the primary antibody were visualized with an alkaline phosphatase-conjugated secondary antibody (Promega Corp., Madison, WI). The western blots were photographed, converted to "tif" files and analyzed using Scion Imaging software available on the NIH web site.

Results

Tiron depressed baseline developed pressure compared to control (79 ± 4 vs. 117 ± 7 mm Hg, respectively, p<0.05). Otherwise, there were no baseline differences in developed pressure between any groups. Recovery of developed pressure during reperfusion was no different between any of the treatment groups and control (Table 3.1).

We observed a marked degradation of troponin I at the end of twenty minutes of ischemia characterized by three degradation bands, designated degradation product 1 (DP 1, slowest electrophoretic mobility), 2 (DP 2) and 3 (DP 3, fastest electrophoretic mobility) (Fig. 3.1). Treatment with the AOXs, Tiron or NAC, did not prevent this
degradation (Fig. 3.2). However, analysis of the bands revealed a pronounced change in the pattern of degradation. Treatment with either AOX resulted in an increase in DP 1 (Fig. 3.3). Treatment with Tiron resulted in a decrease in DP 2 (Fig. 3.3). Treatment with NAC also resulted in a decrease in DP 2 (Fig. 3.3), though it did not reach the level of statistical significance. DP 3 was unchanged by AOX treatment (Fig. 3.3). An additional band appeared in some of the Tiron treated hearts at the end of ischemia (Fig. 3.2); it did not reach statistical significance using densitometry analysis. Because the decrease in DP 2, compared to untreated hearts, observed in the NAC treated group was not proportional to the increase in DP 1, the total degradation in the NAC treated groups was greater than in untreated hearts (Fig. 3.3).

Discussion

The results demonstrate that this model of global myocardial ischemia is associated with significant degradation of troponin I during ischemia. Treatment with the antioxidants Tiron or NAC prior to ischemia resulted in an altered pattern of degradation during ischemia compared to untreated hearts. Treatment with NAC prior to ischemia led to increases in the total amount of Troponin I degradation compared to untreated hearts. None of the AOXs given during baseline or upon reperfusion had an effect on the total amount or pattern of troponin I degradation at the end of reperfusion.

Both Tiron and NAC, when given prior to the onset of ischemia, resulted in similar changes in degradation pattern, namely an increase in DP1 and a decrease in DP 2. This suggests that ROS play a role in the fragmentation of troponin I, which may explain, in part, their ability to decrease the Ca$^{2+}$ sensitivity of myofilaments (87). It is tempting to hypothesize that a two-step process occurs, in which Troponin I is initially
degraded, in a non-oxidant mediated fashion to DP 1. A second, oxidant-mediated, degradation could occur leaving DP 2. Treatment with AOX could have blocked this step leading to an increase in DP 1.

It is also possible that DP 2 is degraded independently from DP 1 in an oxidant-mediated fashion. Blocking this independent pathway with AOX may have increased the amount of native troponin I susceptible to other proteases, such as calpain (85), that may be responsible for the formation of DP 1. At this point, there is no evidence to support a particular hypothesis.

Oxidant stress increases the rate of enzymatic proteolysis (64). Following oxidant exposure, the ubiquitin-ATP dependent pathway plays only a minor role in proteolysis (64, 80), which is chiefly carried out by the proteasome (64). The partial denaturation of the native protein and an increase in hydrophobicity appear to be the oxidant modifications responsible for proteasome mediated degradation (94, 184). Treatment with AOXs may have decreased the oxidant modification, leading to a decrease in proteasome mediated degradation, which altered the degradation pattern.

However, the fact that treatment with NAC prior to ischemia led to increases in the total amount of tropinin I suggests that the ROS generated during ischemia may actually play a protective role in terms of susceptibility of troponin I to degradation. As stated above, oxidant stress is associated with increases in proteolysis (61). However, it is possible that the low levels of oxidants produced during ischemia (19, 235, 130), as opposed to reperfusion, may actually inhibit protein degradation. For example, one
The glutathione system is one of the primary ways in which a cell can reduce oxidized proteins, and return them to their native condition (111). NAC functions as a donor for reduced glutathione (82). This would theoretically augment the action of the glutathione system and increase the reduction of oxidized proteins. This could then expose more protein to enzymatic degradation and explain the increases in tropopnin I degradation observed in the NAC treated hearts.

It is currently accepted that AOXs attenuate post-ischemic dysfunction (27). Therefore, it was somewhat surprising that we did not observe any improvement in post-ischemic function with AOX treatment in this study. However, the buffer-perfused rat heart (55, 222), as well as other models (208, 225, 226), have previously shown a variable response to AOXs as protective agents against myocardial stunning. Despite the presence of the characteristic ROS burst upon reperfusion (89), the buffer-perfused, isolated heart may not be a good model for studying the effects of AOX on post-ischemic contractile dysfunction following global ischemia over this time frame.

If troponin I degradation is the final pathway leading to post-ischemic dysfunction, the lack of functional improvement in our model is not surprising given that the total amount of troponin I affected was either unchanged or increased by AOX treatment. The fact that function was similar between NAC treated and untreated hearts despite differences in total troponin degradation suggests that the degree of post-ischemic dysfunction is not determined solely by troponin I degradation. Because post-ischemic...
function was not improved with AOXs in our model, it is still unclear if ROS lead to stunning via degradation of troponin I. Further studies in a model that is amenable to protection by AOXs are warranted.

A second unexpected finding was that degradation occurred during ischemia. This is in contrast with the findings of Gao et al. (85) who showed no degradation during ischemia and marked degradation during reperfusion. The degradation we observed during ischemia was probably not secondary to infarction as this model is not associated with any increase in CK release, although histologic studies have not been performed.

Lack of degradation during reperfusion has been seen by others (91) and attributed to an inability of the antibody used to recognize the degradation product. This is also the most likely explanation in our case. The degradation products observed during ischemia most likely underwent further proteolysis with loss of the epitope recognized by our anti-Tnl antibody.

In conclusion, it is demonstrated that in certain models of myocardial stunning significant troponin I degradation may occur during ischemia in the absence of infarction. The results presented suggest that the ROS generated during ischemia are involved in the degradation. However, in contrast to the initial hypothesis, the results suggest that low levels of ROS may actually play a protective role.
Table 3.1: Developed pressure at the end of reperfusion expressed as the fraction of baseline developed pressure. Mean ± SEM. (n=5-8 for each group) p=0.18 ANOVA

<table>
<thead>
<tr>
<th>Group</th>
<th>Developed Pressure (fraction of baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>NAC Pre</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>Tiron Pre</td>
<td>0.51 ± 0.1</td>
</tr>
<tr>
<td>NAC Post</td>
<td>0.60 ± 0.1</td>
</tr>
<tr>
<td>Tiron Post</td>
<td>0.39 ± 0.1</td>
</tr>
</tbody>
</table>
Figure 3.1: Immunoblot of Troponin I at various stages of the perfusion protocol (as described in Methods). Demonstrates the appearance of three degradation products, labeled from slowest to fastest electrophoretic mobility DP1, DP 2 and DP 3.
Figure 3.2: Immunoblot of Troponin I performed in AOX treated and untreated hearts at the end of ischemia. Hearts were either untreated or treated with AOXs prior to the onset of ischemia. Band A was a poorly visible, inconsistent band appearing in some of the Tiron treated hearts.
End of Ischemia Degradation

Figure 3.3: Densitometric analysis of Troponin I degradation products at the end of ischemia. Data was expressed as the percent of total Troponin I (the sum of the native troponin I band and all degradation products) that was composed of each degradation band. Total degradation was determined by calculating the percent of Total Troponin I that was made up of degradation products. Values are means ± SEM (n=4-5). * p < 0.01 compared to untreated from same group. ** p< 0.03 compared to untreated from same group. ANOVA with Tukey's post hoc.
CHAPTER 4

Threshold Flow After Global Ischemia to Improve Post-ischemic Myocardial Function and Bioenergetics

There are approximately 300,000 cardiac arrests per year in the United States (104). Resuscitation attempts are usually unsuccessful. Even when there is a return of spontaneous circulation (ROSC) there is often a period of myocardial dysfunction (90,132). This phenomenon of normal coronary flow and depressed function is referred to as myocardial stunning (34,27).

Current resuscitation guidelines call for closed chest compressions, in an effort to provide blood flow to vital organs (1). It is hoped that this low flow will provide enough $O_2$ to extend organ viability during the period of arrest. With respect to the heart, it is hoped that CPR generated coronary flow will attenuate myocardial stunning, and thereby improve perfusion to all organ systems during the post-resuscitation period.

It seems intuitive that any decrease in total ischemic time would translate into improved post-ischemic function. However, it has been suggested that low levels of flow may actually be detrimental compared to continuing complete ischemia through decreasing intracellular pH and metabolite washout (137). Although this has been well studied in the brain and found not to occur in that tissue (137), the effects on the myocardium are less well understood. It has been shown that, in the myocardium,
hypoxia results in more contractile dysfunction than ischemia, probably through washout of metabolites (169, 202). Sufficiently low levels of flow may mimic the hypoxic situation and cause worsening post-ischemic function.

In addition, the effects of low flow on ROS generation are unclear. A burst of ROS upon reperfusion is a well-described phenomenon (207, 250). Perhaps a gradual increase in O$_2$ tension may attenuate the burst of ROS typically seen during immediate postischemic time at normal pressure reperfusion. On the other hand, ROS are also generated during myocardial ischemia (234). It is also possible that low flow perfusion will result in an O$_2$ tension that does not decrease the reducing pressure within the cell, yet provides substrate for production of ROS, leading to an overall increase in the amount of ROS generated.

Although Takeo et al. (221) demonstrated improved post-ischemic function with reduced flow rates following global ischemia vs. immediate ROSC, they studied flow rates that were greater than those generated by standard CPR. We have therefore addressed the question of whether shortening complete ischemic time with levels of coronary flow similar to those generated by CPR is superior to maintaining complete ischemia until normal flows can be generated. We hypothesized that a period of low flow perfusion after complete ischemia and before reperfusion would improve left ventricular function (LVF) and bioenergetics compared to hearts that received prolonged complete ischemia prior to normal pressure reperfusion.
Methods

Preparation and Isolated Heart Perfusion

Male Sprague-Dawley rats weighing 350-450 g supplied by Harlan (Indianapolis, IN) were used in accordance with guidelines of the National Institutes of Health (NIH publication No. 85-23, revised 1985) and the approval of the Ohio State University Laboratory Animal Resources Committee. Rats were anesthetized using an intraperitoneal injection of sodium pentobarbital (50-65 mg/kg), tracheotomized and ventilated mechanically (Harvard Apparatus, Southnatick, Mass). Heparin (1000 U/kg) was administered through the right superficial jugular vein.

The heart was exposed and a cannula (outside diameter of 3-4 mm) was placed into the aorta and secured with a suture. Hearts were immediately perfused, in situ, with Krebs-Henseleit bicarbonate buffer (1.25 mM CaCl₂, and 5.5 mM glucose) with the addition of 0.2 mM caprylic (octanoic) acid at a constant pressure of 85 mm Hg. Buffer was bubbled with 95/5% O₂/CO₂ to attain a pH of 7.4.

The heart was excised from the chest and transferred to a Langendorf apparatus with continuous perfusion. Perfusion pressure was maintained at 85 mm Hg with 37° C Krebs-Henseleit buffer. A water-filled latex balloon, attached to a pressure transducer, was inserted through the left atrium into the left ventricle for measurement of left ventricular (LV) pressure. The heart was submerged in a jacketed, temperature-controlled glass chamber and allowed to equilibrate for 15 minutes. LV end diastolic pressure was maintained at 5 mm Hg. A heart performance analyzer (Digi-Med, Micro-
Med, Louisville, KY) analyzed the signal from the pressure transducer. The following measurements were recorded: LV developed pressure, LV end diastolic pressure (EDP), LV dP/dt max, and LV negative dP/dt max.

**Perfusion Protocol**

The perfusion protocol modeling cardiac arrest with subsequent resuscitation and ROSC was developed. Baseline perfusion at 85 mm Hg with oxygenated buffer was maintained for 10-15 minutes. This was followed by a period of 15 minutes of complete ischemia, followed by a 5-minute period of low flow perfusion with oxygenated buffer and then thirty minutes of reperfusion at baseline pressures. During the low flow (LF) period, hearts were divided randomly to receive 0, 1, or 10% (n=11, 8 and 8 respectively) of their baseline flow and referred to as 0% LF, 1% LF and 10% LF, respectively.

**Metabolite Analysis**

Additional experiments, separate from those done to determine functional results, were performed using identical perfusion protocols to allow determination of bioenergetics. Hearts were freeze-clamped at the end of the baseline, complete ischemia, low flow ischemia and reperfusion periods (n=8 for all groups except, 10% LF end of reperfusion, n=6; and 1% LF end of reperfusion, n=7). They were freeze dried and stored at -80° C. Metabolites were extracted in 0.6 N perchloric acid under liquid N\textsubscript{2} and neutralized with NaCHO\textsubscript{3}. Neutralized extracts were analyzed spectrophotometrically for phosphocreatine (PCr), ATP, creatine (Cr) and inorganic phosphate (Pi) using standard methods (22, 214) The phosphorylation potential [PCr]/[Cr][Pi], the principal determinant of the Gibbs free energy of ATP hydrolysis, was calculated.
Data Analysis

Changes in function were normalized by expressing functional values for each heart as a fraction of the steady state baseline value for that heart. High-energy phosphates were expressed as μmoles/gm. dry wt. All values were expressed as mean ± SEM. Significant differences were determined with one or two-way ANOVA, with Tukey's post hoc test. SAS JUMP statistical software package was used to analyze data (SAS JMP, SAS Institute Inc. version 3.2.2). Values of p < 0.05 were considered statistically different.

Results

Functional Results

LV function approached zero within one to two minutes of global ischemia (Figs. 4.1, 2 and 4). All hearts rapidly showed a return of LV function (Figs. 4.1, 2 and 4) and an increase in LV EDP (Fig. 4.3) upon reperfusion. When the entire reperfusion period was considered, the hearts in the 10% LF group had significantly higher recovery of developed pressures (LV systolic-diastolic pressure) compared to both the 1% and the 0% LF groups (Fig. 4.1). There was a similar improvement in dP/dt max (Fig. 4.2); a measure of left ventricular (LV) contractility.

Diastolic function followed a similar pattern. LV EDP, which is inversely related to LV diastolic function, showed a three-fold greater increase in the 1% and 0% groups than in the 10% LF group (Fig. 4.3). The 10% LF group also had improved recovery of dP/dt max (Fig. 4.4), a measure of the ability of the heart to relax.
Bioenergetics during global and low flow ischemia

Taking measurements at various time points before, during and after ischemia gives a dynamic picture of metabolite changes (Figs. 4.5-7). After fifteen minutes of global ischemia, there was a decrease in both ATP (Fig. 4.5) and PCr (Fig. 4.6) from baseline levels, with a concomitant rise in Pi (15.7±1.9 to 52.4±4.2 μmoles/gm dry wt.). During the next five minutes of ischemia, there was a trend toward a continued decline in ATP in the 1% LF and 0% LF groups, and a leveling off of the ATP level in the 10% LF group. (Fig. 4.5)

PCr levels appeared to continue to decline (Fig. 4.6) during the low flow period in the 1% LF and 0% LF group hearts. In contrast, PCr in the hearts that received 10% LF increased from 4.6±1.0 to 12.4±3.9 μmoles/gm dry wt. (p< 0.02) during the low flow period (Fig. 4.6). At the end of the low flow period, phosphorylation potential was also significantly greater in the 10% LF group compared to the 1% and 0% LF groups (Fig. 4.7).

Bioenergetics during reperfusion

At the end of the reperfusion period, ATP and PCr had recovered to baseline levels in the hearts of the 10% LF group (Figs. 4.5 and 4.6). These levels were significantly greater than those in the 1% LF group and the 0% LF group. Interestingly, the ATP level in the 0% LF was significantly greater than in the 1 % LF group (13.2±1.2 vs. 9.4± 1.0 μM/gm dry wt., p=0.01) at the end of reperfusion. There were no differences in PCr between the 0% and 1% LF groups at the end of reperfusion.
Discussion

This study was undertaken to determine if low flow ischemia following global ischemia, as would be expected in a situation of cardiac arrest, resulted in improvements in LV function and cardiac bioenergetics compared to hearts without the presumed benefits of low flow. We have shown that after global ischemia, coronary flows of 1% baseline (levels of flow typically seen during standard closed chest compressions (72, 73, 203)) result in no increase in any of the measures of energy status. By the end of reperfusion, the hearts that had received 1% low flow actually had decreased ATP compared to hearts that had continuous complete ischemia prior to reperfusion. There were no statistically significant changes between these groups in either systolic or diastolic LV function during reperfusion.

In contrast, when global ischemia was followed by 10% baseline flow prior to reperfusion, there were large improvements in both bioenergetics and LV function. By the end of the low flow period, PCr stores were more than double those available prior to initiation of low flow. By the end of reperfusion, both ATP and PCr had returned to baseline levels. Both systolic and diastolic functions were markedly improved in the hearts that received 10% low flow compared to those with continuous total ischemia and those with 1% low flow.

Although the flows that were selected simulate the range of typical coronary flows generated by closed chest compressions (72, 73, 203), O₂ delivery must also be considered. In vivo, O₂ in the blood is both dissolved in solution and carried by hemoglobin. In this model, O₂ is delivered only in dissolved form. According to Henry's law, the dissolved [O₂] is equal to PO₂ x 0.003 ml O₂ / 100 ml. In our study, perfusate

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PO2 was 600 mm Hg, leading to an O2 content of 1.8 ml O2/100 ml. This is compared to 20.6 ml O2/100 ml, the normal O2 content in the rat (4). This would mean that, in terms of O2 delivery, the 10% LF group is approximately equivalent to CPR delivering 1% flow of oxygenated blood.

It has been postulated that the low flow states observed during chest compressions may actually be detrimental. Studies comparing hypoxia to ischemia offer evidence for the potential harmful effects of low flow. When compared to ischemic hearts, hearts undergoing hypoxia have demonstrated increased EDP during the insult (169, 202), a sign of diastolic dysfunction. It has also been shown that upon reoxygenation from ischemia or hypoxia, the ischemic hearts had superior return of function and improved bioenergetic profiles compared to hypoxic hearts (169, 202). It was hypothesized that this was due to washout of diffusable catabolites during the hypoxic flow (169, 202). The low coronary flows associated with CPR may maintain a state of hypoxia but still be able to wash out metabolites and mimic these findings.

In contrast, it has been shown that graded reperfusion following ischemia, at flows below baseline, improves postischemic function compared to immediate reperfusion at baseline levels (160, 221). However, the lowest reperfusion flow tested was 10% of baseline and offered only minimal improvements in post-ischemic function. The maximal improvement in post-ischemic function was seen with flow rates of 40% baseline values (221), a level of flow unlikely to ever be achieved with standard CPR.

The bioenergetics data presented imply that there is a threshold level of post-ischemic low flow below which energetics worsens and above which they improve. One percent baseline flow following ischemia was not sufficient to support energy production...
during the low flow period. Furthermore, despite a 33% increase in the duration of complete ischemia, ATP levels during reperfusion were greater in 0% low flow hearts than in 1% low flow hearts. We speculate that flows of 1% baseline, especially in our model in which this level of flow delivered only 10% of the O₂ that would be present in blood, may have represented the situation seen in the studies comparing hypoxia to ischemia (169, 202). Hearts exposed to 1% LF were not able to support aerobic respiration, yet may have been able to wash out ATP catabolites (202).

Conversely, 10% low flow provided enough substrate to support aerobic respiration during the low flow period and overcome any wash out of diffusible catabolites. This is a situation similar to the graded reperfusion seen by Takeo et al. (221). In that study, reduction in calcium overload was thought to be the mechanism of action. Though we currently have no supporting data, it would seem that the ability to maintain normal amounts of ATP and PCr requires well functioning mitochondria. Therefore, we hypothesize that this level of flow results in improved post-ischemic mitochondrial function.

It has been shown that insufficient energy production is one of the main determinants of myocardial ischemic injury (116, 236). The relationship between energetics and post-ischemic function is less certain. Several studies support a relationship between cardiac function and a variety of energetic parameters including 1) phosphorylation potential (52), 2) PCr levels (142), and 3) the ratio of PCr/ATP (127). Other groups have evidence against such a relationship (109, 205, 219). Hoerter et al. (109) showed that despite depletion of PCr to 15% of normal and ATP to levels undetectable by nuclear magnetic resonance (NMR), normoxic hearts were still able to
develop 65% of their normal systolic pressure and had no change in diastolic pressure. Saupe et al. (205) monitored energetics while decreasing perfusion pressures slowly, and showed that the decrease in myocardial contractility preceded any change in cardiac energetics.

Our data supports the hypothesis that changes in function are not dependent on changes in energetics. However, a partial, causal relation should not be entirely dismissed. Although Hoerter et al. (109) showed a fall in high-energy phosphates out of proportion to the fall in cardiac function, it cannot be excluded as one cause of the contractile failure that did occur. Similarly, the experiment by Saupe et al. (205) showing that a fall in cardiac function could precede a change in bioenergetics does not exclude any link between energetic status and function. In an experiment with similar time resolutions, but a more rapid onset of ischemia, He et al. (107) showed that changes in cardiac energetics preceded a decrease in systolic function.

In this study, despite baseline levels of PCr at the end of reperfusion in the 10% low flow group, phosphorylation potential was still significantly reduced since the [ATP] hadn’t fully recovered. This could be a critical value related to function (52) and may explain the sub-baseline level of LV function despite normal PCr levels in this group. In addition, function was greatly improved compared to the 0% and 1% group. It is possible that the functional improvement that was seen is due to the increase in energy availability and that other factors, such as injury to the contractile apparatus (172), may prevent complete return of LV function.

I therefore believe that the improved function seen during reperfusion in the 10% low flow group is partially explained by improved bioenergetics. There are other
possible alternative or concurrent explanations. Among these is improved calcium homeostasis, either through maintenance of ionic gradients (which may be secondary to improved energy availability) and/or SR membrane integrity as well as protection of contractile protein function (172). The precise mechanism by which low flow perfusion following ischemia leads to changes in bioenergetics as well as function remains a matter of speculation.

The major limitation of the study is the use of an ex vivo preparation to model cardiac arrest. One of the primary abstractions was the use of oxygenated salt solution instead of blood as a perfusate. This presumably introduced several changes from the in vivo setting, including the lack of white blood cells and inflammatory mediators, changes in dynamics of fluid flow and, as discussed earlier, changes in oxygen content and delivery. However, in the reperfusion time frame considered in this study, it has been shown that neutrophils play little or no role in stunning (29, 182).

In conclusion we have shown that, in the perfused heart, low flow ischemia of 1% baseline flow (0.1% baseline O₂ delivery) following complete ischemia and prior to reperfusion worsens cellular bioenergetics and gives no significant improvement in post-ischemic function. However, post-ischemic flows of 10% baseline (1% baseline O₂ delivery) prior to reperfusion improve both bioenergetics and LV function. Therefore, there is a threshold level of flow and/or oxygenation between these two levels, below which bioenergetics are worsened without LV function improvement and above which they are both improved.

It is possible that this threshold represents the point above which oxidant stress is attenuated and below which oxidant stress is accentuated. That is, perhaps above some
threshold $O_2$ delivery the reducing environment that is the result of ischemia is dissipated but the relative lack of $O_2$ compared to reperfusion at normal pressures supresses the formation of ROS. Below the threshold flow, the reducing pressure generated during ischemia would not be resolved and the additional $O_2$ present, compared to ischemia, would provide substrate for increased generation of ROS.

These findings support the hypothesis that standard CPR can deliver enough $O_2$ to improve postischemic LVF and bioenergetics. However, sub-optimal technique (1) fixed coronary stenosis (131, 176) and prolonged resuscitation (72, 79) can all lead to decreases in CPR-generated flow. It is therefore possible that CPR will generate flows that fall below the critical threshold and could interfere with the bioenergetic recovery of the post-ischemic myocardium.
Figure 4.1: Plot shows the time course of Developed Pressure. Values are mean ± SEM. *P<0.0001 (ANOVA over the reperfusion time period) 10% LF vs. 1% and 0% LF
Figure 4.2: Plot shows the time course of dP/dt Max. Values are mean ± SEM. *P<0.0001 (ANOVA over the reperfusion time period) 10% LF vs. 1% and 0% LF.
Figure 4.3: Plot shows the time course of End Diastolic Pressure. Values are mean ± SEM. *P<0.0001 (ANOVA over the reperfusion time period) 10% LF vs. 1% and 0% LF.
Figure 4.4: Plot shows the time course of $-dP/dt$ Max. Values are mean ± SEM.
*P<0.0001(ANOVA over the reperfusion time period) 10% LF vs. 1% and 0% LF
Figure 4.5: Effects of perfusion protocol on ATP levels. Values are mean ± SEM. *P<0.001 vs. time matched Control. †P=0.01 vs. time matched 1% Low Flow. ‡P<0.01 vs. time matched Control. (Anova with Tukey’s post-hoc)
Figure 4.6: Effects of perfusion protocol on PCr levels. Values are mean ± SEM. 
*P<0.001 vs. time matched Control and 1% Low Flow. ‡P=0.02 vs. 15 minute ischemia. 
†P<0.01 vs. time matched Control and 1% Low Flow. (Anova with Tukey's post-hoc)
Figure 4.7: Effects of perfusion protocol on Phosphorylation Potential. Values are mean ± SEM. *P<0.01 vs. time matched Control. †P=0.001 vs. time matched Control.
†P<0.01 vs. time matched Control. (Anova with Tukey’s post-hoc)
CHAPTER 5

TENSION-TIME INDEX, FATIGUE AND MUSCLE ENERGETICS IN ISOLATED RAT DIAPHRAGM

The tension time index (TTI) can be defined as the average pressure developed by a rhythmically contracting muscle, expressed as a fraction of baseline maximum tetanic tension. Therefore, TTI increases directly with duty cycle (time of contraction / total contraction-relaxation time) and the average force generated during contraction. The TTI is believed to be a key determinant of the development of skeletal muscle fatigue (20, 206), the rate of energy consumption (60) and muscle blood flow (21), being most relevant to conditions of isometric or near-isometric contractions (50). The origins of the tension time index concept arose from the observation that a primary determinant of myocardial $O_2$ consumption in the heart is the product of heart rate times the accumulated area under the systolic pressure curve (204). Its application to the study of human skeletal muscle fatigue, in a wide variety of muscle fiber types, has shown that voluntary contractions with TTI values 0.15 or more, ultimately result in muscle fatigue (20, 206).
Despite a relatively wide acceptance of the TTI concept to understanding fatigue and energetics in intact, perfused models of muscle contraction, it has generally not been applied to understanding the behavior of isolated muscle preparations. For example, in vitro, non-perfused rat diaphragm muscle (when studied at stimulation frequencies of 20-30 Hz) cannot sustain contractions with average TTI values even close to 0.15. In fact, within four minutes, force development falls well below this level (81, 240, 241).

Mechanisms that might be responsible include potential influences of tissue hypoxia in isolated muscle. Without perfusion, and with diffusion distances in excess of 150 microns, cellular hypoxia could occur as TTI increases, though this has never been measured directly. In addition, the loss of neuromuscular adaptation makes isolated artificially stimulated muscle more susceptible to losses in force at a fixed stimulation frequency. In intact neuromuscular systems, where the TTI concept has proven most valuable, additional force reserves are recruited as muscles fatigue by increases in stimulation frequency and/or recruitment of new motor units. Finally, in vitro, non-perfused muscle fibers may be more susceptible to mechanisms of "high-frequency fatigue" (121) because of delayed washout of metabolites and compromised transmembrane ion gradients, which are believed to be responsible for this phenomenon (121).

The primary objective of this study was to re-explore the applicability of the TTI concept to both the metabolic and contractile responses of in vitro muscle fatigue using isolated rat diaphragm. To accomplish this, a new experimental paradigm was developed allowing ongoing TTI to be clamped at a single value as the muscle fatigues. This was done by making continuous adjustments in duty cycle or stimulation frequency. Using
this technique, the following hypotheses were tested: 1) The extent of contractile fatigue at a given TTI is dependent on stimulation frequency in an in vitro setting, and 2) the metabolic state of the muscle during fatigue is dependent on the TTI and is independent of the stimulation frequency or duty cycle.

The results are consistent with the concept that the TTI is a predominant variable in determining the metabolic and contractile responses to fatiguing stimulations in isometric, isolated diaphragm muscle. Furthermore, within the time frame and conditions of these experiments, the phenomena of low- and high-frequency fatigue appear to be indistinguishable. Finally, the results suggest that much of the disproportionate loss of force during fatigue in isolated muscle at low stimulation frequencies is due to the inability to recruit additional force reserves by increased activation, which would be available in intact neuromuscular control systems.

**Methods**

**In vitro diaphragm muscle strips**

Adult Sprague-Dawley rats (300-500 g) were anesthetized with intraperitoneal pentobarbital sodium, tracheotomized, and mechanically ventilated. In each animal the entire diaphragm was dissected out, with associated ribs and central tendon intact, and immediately placed in physiologic salt solution (251) and bubbled with 95% O₂-5% CO₂. The diaphragm was gently cleaned of blood, and excess ribs and fat were trimmed. Strips, 5-10 mm wide, were then cut along the plane of the muscle fibers, and included a portion of the central tendon and the associated rib or ribs. The muscle strips were then placed in tissue baths containing 25 ml of physiologic salt solution, continuously bubbled with 95% O₂-5% CO₂ and maintained at 37° C. Each strip was mounted vertically in a
tissue bath with the central tendon superior and attached to a force transducer. The force transducer was attached to a micropositioner. The strips were positioned between two circular platinum wire stimulating electrodes.

**Contractile Properties**

After mounting, the maximum required stimulation current and the preload required to reach optimal length ($L_0$) were determined using twitch contractions. All stimulations were done at supramaximal voltages and at $L_0$. Force-frequency relationships were determined with 400 ms trains at frequencies of 20, 30, 40, 50, 60, 80, 100 and 150 Hz with 20 seconds between train stimulations.

**Fatigue protocol**

A new model of fatigue was developed in which the muscles were stimulated to contract at a single TTI throughout the fatigue protocol. The TTI was determined as a fraction of the maximum tetanic force generated by the muscle during the preliminary force-frequency measurements. For the purposes of this study, the TTI was always controlled by making continuous adjustments in duty cycle, though the system allowed for maintaining TTI with stimulation frequency as well. Since most studies of isolated diaphragm fatigue use stimulation frequencies of around 20 Hz, preliminary experiments were performed to determine the TTI that could be sustained for four minutes at this frequency. At 20 Hz, diaphragm muscles could continue to contract for 4 minutes at a TTI of 0.08 or below. When target TTI values were set higher than 0.08, duty cycle had to be increased to 1.0 (i.e. continuous contraction) during the fatigue experiment. When this occurred, the muscle quickly lost active tension and could not sustain the target TTI. Therefore 0.07 was set as the target and the maximum frequency used was 25 Hz.
To control TTI, the force measurements were digitized at > 1 kHz (Strawberry Tree, Inc.) and the digitized values were processed by data acquisition software (Workbench 5 for Windows, Strawberry Tree, Inc). Using a summing amplifier, a voltage equivalent to the preload on each muscle was then subtracted from the total force signal to determine a value for active force. A moving average of active force, over time, was then determined by calculating an ongoing exponential, weighted mean of the signal, with a time constant set at 3 sec. This signal was continuously displayed on a computer screen that was used as an oscilloscope. The target TTI (i.e. 7% of baseline max tetanic force at 150 Hz) was then identified on the screen, and during the fatigue run, ongoing adjustments in duty cycle were continuously made to keep the muscle contracting with small oscillations around the target TTI. To determine the influence of stimulation frequency on the energetics and fatigability of the muscles, four muscles in each animal were studied in each of the four baths, at 25, 50, 75 and 100 Hz. Other than frequency of stimulation, all tissues were treated identically. In every study, the frequency of contractions was kept at 1 per second.

**Experimental protocol**

Approximately ten minutes after L₀ was determined, a force frequency determination was done on all muscles (approximately 30 min. equilibration at 37°C). The bath solutions were then changed. After approximately 15 minutes of rest, the muscles underwent the fatigue protocol, one strip at a time. The order in which the different groups were fatigued was randomly determined. Twenty seconds after the fatigue protocol ended each of the tissues was subjected to stimulations at 20 and 150 Hz to determine the extent of fatigue at these frequencies. In a separate group of
experiments the muscle strips were immediately freeze-clamped upon completion of the fatigue protocol (usually within 1-2 sec of the last fatigue contraction). The technique ensured a clean piece of diaphragm without tendon or ribs, comprising 4/5 of the center of the diaphragm strip. Frozen tissues were stored in a -80° C until analysis. A separate set of control diaphragm strips was also studied. These strips were unstimulated.

**Analysis of muscle high-energy phosphate status**

The frozen tissues were ground with mortar and pestle under liquid nitrogen. The high energy phosphates and other metabolites were immediately extracted in 0.6 N perchloric acid and neutralized with NaHCO₃ to pH 7.0. Neutralized extracts were then analyzed spectrophotometrically for PCr, ATP, Cr and Pi using standard methods (22, 214). Phosphorylation potential was calculated as PCr/(Cr • Pi).

**Results**

Baseline tension generation for all tissues was 9.1± 0.5 N/cm² at 20 Hz and 26.4 ± 1.1 N/cm² at 150 Hz. There was no significant difference between groups in baseline tension at either stimulation frequency, and no individual group varied from the overall mean by more than 10% at either frequency. Following the fatigue protocol there was a decline in force generation at both 20 Hz and 150 Hz (Fig 5.1). There was no difference between experimental groups at either frequency. Therefore, muscles fatigued at the same TTI, but at varying frequencies of stimulation, showed no difference in the extent of contractile fatigue at low or high frequencies, when measured 20 seconds after the fatigue runs.

Energetics data is summarized in Table 5.1. The total ATP present in the tissue immediately following the fatigue protocol was unchanged from baseline levels. In
contrast, both PCr and phosphorylation potential fell from unstimulated levels. There
were, however, no differences between the groups fatigued at different frequencies.
Interestingly, there was no change in Pi in fatigued tissues when compared to baseline.

The duty cycle that was needed at the end of the fatigue protocol to maintain
constant TTI was inversely related to stimulation frequency (Fig. 5.2). This was expected
as the tension generated increases with the frequency of stimulation.

Discussion

These results demonstrate that TTI is a predominant factor in determining the
metabolic and contractile responses of fatiguing, isolated skeletal muscle. Both the
extent of fatigue and the energetic state at the end of fatigue appear to be independent of
the pathway taken to achieve a constant TTI, whether that be by increasing duty cycle or
increasing frequency of stimulation. Although other factors may predominate at more
intense levels of stimulation, within the range studied here, a single variable, TTI, can be
used to predict the fatigue response.

There are several important implications of these findings. First, the results are
clearly in support of the concept that fatigue responses to low stimulation frequencies
(one definition of "low frequency fatigue") reflect alterations in excitation-contraction
coupling. For example, the very low forces that are generated at the end of a fatigue run
at low frequencies (e.g. 20 Hz) do not reflect the capacity of the muscle to sustain
contractile force. In contrast, the identical TTI could be easily maintained at a duty cycle
less than 0.1 when the muscle was stimulated at the highest frequencies (100 Hz).
Therefore, very large force reserves remain after fatigue at low stimulation frequencies
that simply cannot be recruited because of limitations to EC coupling at this frequency.

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This inherent limitation is compensated for in an intact muscle with normal neuromuscular adaptive reflexes intact. Therefore, if one wanted to determine the maximal sustainable TTI for \textit{in vitro}, isometrically, contracting tissue, some frequency considerably higher than 20 or 30 Hz would be needed. There must be an "optimal" stimulation frequency in this model, which would result in a maximum TTI value that could be maintained over a 4 min. fatigue trial. This optimal stimulation frequency could not be determined by these experiments but would be of some value for future studies.

Second, there is no clear distinction between low and high frequency fatigue at this level of stimulation. Prior to performing the study, we hypothesized that at very high frequencies there would be a greater degree of contractile fatigue and an inability to sustain the target TTI because of a presumed recruitment of high-frequency fatigue mechanisms. When muscle is stimulated at high frequency, a mechanism of fatigue (not present at low frequencies) is observed which involves increases in extracellular $\text{K}^+$, particularly in the t-tubules (121). It has been shown that high frequency stimulation results in greater degrees of fatigue than low frequency stimulation (35, 198). However, no evidence for such limitations was seen in this study, thus disproving our hypothesis.

No doubt, high frequency fatigue does exist. However, it is generally studied under conditions of prolonged trains of stimulation (on the order of tens of seconds) (121, 198), rather than the short, repetitive stimulations such as occur during the normal activity of the diaphragm. It is therefore possible that, although others have reported increases in fatigue with high frequency even when the protocol consists of short, repetitive, contractions (35), our model does not manifest the mechanisms proposed to explain high-frequency fatigue.
Third, diaphragm preparations are frequently utilized to study the influence of drugs or treatment paradigms on skeletal muscle fatigability. A practical problem that our laboratory and many others have dealt with is the fact that many drugs and treatments affect the relationship between baseline force and frequency of stimulation (69, 133). Therefore, comparison of fatigability between groups is compromised because muscles under different treatments may be contracting with greater or lesser force or greater or lesser energy requirements throughout the fatigue protocol at a given stimulation frequency. This problem is rarely addressed. One method, used by Khawli et al. (133), was to match the relative force of the two muscles being compared by adjustments in stimulation frequency. The results of the present study suggest that such a method is a perfectly appropriate approach to the problem since frequency has little effect on the fatigue process within these ranges of TTI. However, an even better approach would be to match TTI throughout the fatigue protocol as we have done in this model. This approach would compensate for any transient changes in force-frequency (e.g. via potentiation mechanisms) that are known to evolve during the fatigue process and could evolve differently with various treatments.

Insights into the fatigue process

Fatigue is thought to occur secondary to multiple factors, including increases in inorganic phosphate (56), decreases in intracellular pH (56), alterations in Ca\(^{2+}\) cycling (14) and many others. We found no change in \([\text{Pi}]\) between stimulated and unstimulated muscles. This rules out increases in Pi as a mechanism in our model. Whatever the prevailing mechanism of fatigue in this model, it is apparently very closely associated with some aspect of TTI. But why?
One possibility is that TTI is linked to the fatigue process through the turnover of intracellular \([\text{Ca}^{2+}]\) (150). For example, Chin et al. (49) found that the total amount of \(\text{Ca}^{2+}\) cycled over the fatigue time, the so-called "Calcium-Time Index" (CTI), was the key factor in the development of fatigue. It is compelling to speculate that the CTI is determined by the TTI and that this is the mechanism by which TTI and fatigue are related. This idea will be returned to later in the text.

Another link between fatigue and TTI is its close association with the energetic cost of contraction. In isometrically contracting muscle, at a constant activation, the primary determinant of energy utilization is the tension-time product, whereas with shortening contractions additional energy utilization is expended in the performance of external work, over and above the tension developed (112). Our results regarding the energy status of the muscles at the end of fatigue are entirely consistent with the energetic cost of contraction being determined by TTI. It seems highly likely that if the extent of the loss of contractile function with fatigue is due to feedback inhibition from the products of metabolism, such as \(\text{H}^+\) or \(\text{Pi}\), that these would be determined by the net energy balance, which our results demonstrate are determined by TTI.

As presented, the energetics data represents the sum total of all energy-consuming and energy-producing processes. It would seem that the simplest explanation for the equivalent energetic status of all groups is that the energy consumed and the energy produced were individually equivalent between groups.

The energetic cost of contraction is divided between the energy utilized for cross bridge cycling and for \(\text{Ca}^{2+}\) cycling (187). During single twitch contractions ~ 30% of energy usage goes towards \(\text{Ca}^{2+}\) ATPase, and possibly proportionately less than this is
required per stimulation during tetanic contractions (187). Again, the simplest explanation is that the costs of Ca\(^{2+}\) cycling are identical between groups at constant TTI, as are the costs of cross bridge cycling. As speculated earlier, TTI may determine the CTI. If so, the total amount of Ca\(^{2+}\) to be cycled would be identical between groups, and therefore Ca\(^{2+}\) cycling costs would apparently be the same. If \([Ca^{2+}]\) determines the number of cross bridge interactions, then TTI would theoretically determine energy costs as well.

This is supported by calculations of the approximate number of individual twitch stimulations per second (averaged over contraction and relaxation) at the end of the fatigue runs. The total number twitches per second for stimulation frequencies between 25-100 Hz varied only between 7.5-9.5 twitches/second, independent of frequency, duty cycle or other variables. If one makes the simplistic assumption that the cross bridge cycling/twitch and the Ca\(^{2+}\) cycling/twitch are constant during tetanic contractions at different frequencies, then the energetic cost of Ca\(^{2+}\) cycling would be the same between groups.

Interestingly, not only would TTI likely fix energy demand by these mechanisms, but energy supply could be linked as well. Based on the classic work by Chance and Williams (44), respiratory rate is regulated by the ADP available for phosphorylation. Therefore, for a given rate of energy demand (presumably fixed by TTI), there would be a proportional stimulation of mitochondrial metabolism through the rise in [ADP]. How well the ADP-dependent regulation of metabolism applies to \textit{in vivo} conditions has recently been questioned (15, 139).
However, proposed pathways of parallel metabolic activation have been proposed, in which both ATP consumption and production are directly activated by an external factor (139). In contracting muscle one potential candidate for this factor is Ca$^{2+}$ (36, 103, 139). This leads to the interesting possibility, as speculated earlier, that in our model the link between TTI and the process of fatigue is related to Ca$^{2+}$ flux. Identical Ca$^{2+}$ fluxes would, theoretically, have the same cycling cost, cause the same amount of cross bridge cycling and, if parallel activation occurs, stimulate respiration equally between groups, thus resulting in a matched metabolic costs of contraction.

In conclusion, we have shown that the amount of fatigue developed by repetitive, isometric contractions is very carefully matched to TTI in this range of stimulation and is independent of the frequency of stimulation. Likewise, TTI appears responsible for determining the energetic status of the cell during the fatigue process. These findings allow the development of a novel experimental model of in vitro fatigue in which energy state may be matched between study groups.
Table 5.1: Energetic data (in units of μmoles/mg protein, except phosphorylation potential which is in mg/μmoles) measured at baseline, prior to stimulation, and at the end of the fatigue protocol. Mean± SEM. * p < 0.05 ANOVA with Tukey’s Post Hoc. (n=6 for each group)
Figure 5.1: Tension generated at 20 and 150 Hz following the fatigue protocol at the indicated frequency, expressed as a fraction of the baseline tension. Mean ± SEM (n= 6 for all groups except 100 Hz in which n=5).
Figure 5.2: Duty cycle needed to maintain TTI of 0.07, measured at the end of the fatigue protocol. Curve represents data fit to an exponential decay, described by the included equation. Means ± SEM. (n= 8 for all groups)
CHAPTER 6

EFFECT OF ANTIOXIDANTS ON DIAPHRAGM FATIGUE

Multiple groups have shown that antioxidant (AOX) treatment is able to attenuate fatigue in skeletal muscle (6, 9, 69, 70, 191, 193). One obstacle in interpreting the data from fatigue/AOX experiments arises secondary to baseline changes in muscle function in the presence of AOX (192). Specifically, it has been shown that tension at low frequencies is depressed in the presence of AOXs (192). One way that this has been compensated for has been by altering stimulation frequencies to match tension between treated and untreated muscles (133). As was discussed earlier, this generates further problems since frequency of stimulation may have independent effects on muscle fatigue (24). Furthermore, any changes in energetic cost between groups because of varying tension-time would confound any study of muscle energetics, an area of particular interest in this dissertation.

A novel model of fatigue was proposed in the prior chapter that appears to have solved these problems. In this study I will compare the effects of a variety of AOXs on fatigue in two models; the first being the standard model currently used in this lab (69), the second being the model suggested in chapter 5. Data is presented showing identical results in each model, further verifying the validity of the novel model.
Methods

In vitro diaphragm muscle strips

Adult Sprague-Dawley rats (300-500 g) were anesthetized with intraperitoneal pentobarbital sodium, tracheotomized, and mechanically ventilated. In each animal the entire diaphragm was dissected with associated ribs and central tendon intact, and immediately placed in physiologic salt solution (69) and bubbled with 95% O₂-5% CO₂. The diaphragm was gently cleaned of blood, and excess ribs and fat were trimmed. Five to ten mm wide strips were then cut along the plane of the muscle fibers, and included a portion of the central tendon and the associated rib.

The muscle strips were then placed in tissue baths containing either ~125 or 25 ml, depending on bath size, of physiologic salt solution and continuously bubbled with 95% O₂-5% CO₂. The strips were mounted vertically in the tissue bath with the central tendon superior, and attached to a force transducer, which was attached to a micropositioner. The strips were positioned between two platinum plates, in the case of the large bath, or two wire electrodes in the case of the smaller baths.

Stimulation

After mounting, the maximum required stimulation voltage and the optimal force-optimal length (L₀) relationship were determined. All stimulations were done at supramaximal voltages and at L₀.
**Force frequency relationships**

Force-frequency relationships were determined by tetanic stimulation of the strips at 20, 30, 40, 50, 60, 80, 100 and 150 Hz. There was an interval of 20 seconds between stimulations. Pulses were of 0.2 ms duration, with a train duration of 400 ms. One twitch stimulation was performed prior to each force-frequency determination.

**Protocol 1**

Approximately ten minutes after \( L_0 \) was determined, a force-frequency determination was done on all baths. The baths were then changed. One had the physiologic salt solution replaced and served as a control, the others had the physiologic salt solution as well as either Tiron (10 mM), NAC (10 mM) or SOD (500 units/ml) added. Approximately thirty minutes later, a second force frequency determination was performed. At that time, the baths were again changed, and the muscles were rested for ten minutes. The strips were then subjected to a fatigue protocol consisting of 4 minutes of intermittent tetanic contractions (20 Hz, 0.33-s train, 1 train/s).

A Gould chart recorder was used in all experiments to record muscle contractions. Max force was defined as the maximal force produced at 150 Hz, on the force frequency relationship determined just prior to fatigue protocol. Percent max tension was defined as the ratio of the tension the muscle was able to produce at the end of the fatigue protocol, divided by max force and multiplied by 100.

**Protocol 2**

Approximately ten minutes after \( L_0 \) was determined, a force-frequency determination was done on all baths. The baths were then changed. One had the physiologic salt solution replaced and served as a control; the others had the physiologic salt solution replaced and served as a control; the others...
salt solution as well as either Tiron (10mM), deferoxamine (100 μM) or MnTMPyP
[Mn(III)tetrakis(1−Methyl−4−pyridyl)porphyrin Pentachloride] (50 μM), a cell membrane
permeable SOD mimic. Approximately thirty minutes later a second force-frequency
determination was performed. At that time, the baths were once again changed, and the
muscles were rested for ten minutes. During this time the frequency required to achieve
40% of the max force, as measured during the last force frequency determination, was
calculated for each muscle strip by considering the change in force frequency to be linear
between 20 and 30 Hz. The muscle strip was stimulated during the subsequent fatigue
run at this frequency. In this way, forces were matched for each tissue.

The new model of fatigue (described in the prior chapter in which the muscles
were stimulated to contract at a single TTI throughout the fatigue protocol) was
employed. The protocol consisted of 4 minutes of intermittent contractions, 1 train/sec,
with frequency of stimulation determined for each muscle individually so that forces
were matched, as described above. Duty cycle was continuously adjusted to maintain
TTI of 0.07.

The duty cycle required to meet a certain TTI is inversely related to the tension
being generated. Since all tissues were being stimulated at matched forces, the duty cycle
at the end of the fatigue run could be used to measure the relative fatigue in each muscle.
The higher the duty cycle, the more fatigued the muscle.

Results

In protocol 1 the amount of fatigue was determined by comparing the amount of
force generated by each muscle at the end of the protocol to the maximum force it
produced prior to the protocol. Antioxidants failed to attenuate fatigue when stimulated
using the standard protocol (table 6.1). Similar results were found when muscle was
stimulated per protocol 2. No AOX had a significant effect on fatigue development, as
demonstrated by the duty cycle required to maintain the target TTI (table 6.1).

**Discussion**

Antioxidants failed to attenuate fatigue in either model. In one sense this is
effectively encouraging, since it demonstrates that the new model gives the same result as the
standard, accepted model. However, the lack of effect in either protocol is in contrast to
almost all previously published reports on the effects of AOX on fatigue (9, 191, 193),
including results from this very lab (70).

When this dissertation work was begun, the original intent was to study chiefly
ROS-induced mechanisms of muscle fatigue. However, the initial experiments, as
presented here, showed no effects of AOXs on fatigue, rendering this line of investigation
less than fruitful.

A prolonged effort was put into trying to determine the reason behind our
inability to reproduce our own results. This included exact duplication of other lab's
protocols (133), alteration of our solutions, trying different manufacturers and
preparations of AOX, using rats of various ages, as well as having several different
people in the laboratory perform the experiments. In all cases, the same result was
obtained: AOXs had no effect on diaphragm fatigue.

Furthermore, as was stated earlier in the dissertation, the initial experiments were
done to study the hypothesis that AOXs attenuated fatigue by preserving high-energy
phosphates. Experiments were carried out, in conjunction with the above fatigue
experiments, to explore this hypothesis. After the fatigue runs the tissues were frozen
and metabolites extracted in perchloric acid and HPLC was used to determine high energy phosphates (224). However, further technical difficulties were encountered as outlined below.

It came to our attention during the study period that the PCr measurements were inconsistent. Namely, the graph of PCr/total protein vs. total protein, which should have yielded a slope of zero, had a negative slope. For example, with other things being equal, if total protein of the sample measured doubled, the other metabolites should have doubled. After extended trouble-shooting, two relevant facts were uncovered. First, the technique that our lab as well as several other labs had been using for perchloric acid extractions did not result in adequate neutralization of the extract (pH <1.0). This can lead to degradation of high-energy phosphates. Second, even when the extract was properly neutralized, a low molecular weight substance, present in the perchloric acid, came off the HPLC column at the same elution time as PCr, and interfered with the HPLC signal.

The end result was that acid hydrolysis of the metabolites had rendered all of the samples unusable for energetic determination. In addition, the additional “acid peak” rendered the technique sub-optimal for further energetic work, even with properly neutralized extracts.

Several positives came out of this trying time. First, I developed a new model of fatigue that will be of use in answering many basic questions regarding fatigue. Second, I developed a new neutralization method, and got several assays for measuring high-
energy phosphates on the spectrometer up and running. These techniques have become standard methods in our laboratory and allowed me to accurately study the relationships between high-energy phosphates in the previously described studies.
Table 6.1: Effect of antioxidants on skeletal muscle fatigue. For protocol 1, measurement is the amount of force each tissue was generating at the end of the fatigue run, expressed as a fraction of the maximum force generated prior to fatigue (n=7 for all groups). For protocol 2, the measurement is the duty cycle required to maintain a TTI of 0.07 at the end of the fatigue run (n=6 for all groups).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Max Force</th>
<th>Treatment</th>
<th>Duty Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34 ± 5</td>
<td>Control</td>
<td>0.48 ±0.06</td>
</tr>
<tr>
<td>NAC</td>
<td>26 ± 5</td>
<td>Deferoxamine</td>
<td>0.47 ±0.06</td>
</tr>
<tr>
<td>SOD</td>
<td>26 ±5</td>
<td>MnTyMNP</td>
<td>0.53 ±0.06</td>
</tr>
<tr>
<td>Tiron</td>
<td>29 ±5</td>
<td>Tiron</td>
<td>0.48 ±0.07</td>
</tr>
</tbody>
</table>
Although the generation of ROS has long been considered a primary factor in the development of post-ischemic contractile dysfunction in the heart, the mechanism by which they exert their effects is poorly understood (27). This dissertation provides evidence that ROS play a complex and perhaps paradoxical role in both high-energy phosphate regulation and the proteolysis of troponin I, both of which are thought to be intimately related to the recovery of post-ischemic function (58, 85). What is perhaps most interesting was the finding that attenuation of ROS at the time of reperfusion, the burst of ROS thought to be most important in development of ventricular dysfunction, had very few if any effects on either high-energy phosphate recovery or troponin proteolysis. In contrast, attenuation of ROS during ischemia, a time when low levels of ROS are believed to be generated (19, 130, 235), had pronounced effects on both processes.

AOX treatment during ischemia inhibited recovery of high-energy phosphates during the reperfusion period compared to untreated hearts. This effect appeared to be secondary to a decrease in efficiency in AOX treated hearts compared to untreated hearts. This implies that ROS generated during ischemia function to increase the efficiency of
contraction of myocardium during reperfusion. This points to a physiologic role for ROS generated during ischemia, which serves to protect the heart during the reperfusion period. The mechanisms by which ROS increase efficiency are unknown, but it is hypothesized that it may be through an activation of protein kinase C, which has been previously observed (177).

The effects of AOX treatment on degradation of Tn I was also limited to those given during ischemia. Tn I breakdown was observed at the end of ischemia, with three distinct degradation products visible. Treatment with the AOX NAC during ischemia actually increased the total amount of degradation seen. Both AOX altered the pattern of degradation. The amount of DP 2, which was the most prominent degradation product observed in untreated hearts, was decreased with a proportional increase in degradation product 1. Although the mechanism by which this shift occurred is not known, it is hypothesized that DPs 1 and 2 are the result of a two-step process. The first non-ROS-mediated process leads to DP 1. This is followed by a second ROS-mediated, degradation leading to DP 2.

Our treatment was not associated with any change in function. However, alteration in degradation pattern may be important in other models since it has been hypothesized that it is not the amount of Tn I degraded that decreases Ca^{2+} sensitivity and leads to ventricular dysfunction, but rather some soluble fragment associated with degradation (91).

There are several weaknesses in the studies described in this dissertation. Some are a result of experimental design. An attempt was made to determine the role of ROS during I/R. This was done indirectly using AOXs. No attempt was made to directly test
the effects of ROS exposure on either high-energy phosphate production or TnI proteolysis. Furthermore, no measurement of ROS generation, either directly with electron spin resonance (ESR) or indirectly through the measurement of fluorescence, glutathione redox state etc. was performed. An assumption was made that our protocol generated ROS and that our AOX treatment attenuated this stress.

A second group of weaknesses revolves around the model of I/R used. First, unlike most other studies that treated the myocardium with AOXs during I/R (27), there was no improvement in post-ischemic function in treated vs. untreated hearts. Although several other groups have seen the same lack of protection with AOX therapy (208, 225, 226), the lack of any improvement in these experiments limits the conclusions that can be drawn regarding the role of ROS in the development of post-ischemic dysfunction. Although the findings are interesting in their own right it cannot be concluded that they play any role in the dysfunction resulting from ischemia and reperfusion. Further experiments, performed in a model known to demonstrate functional improvement with AOX therapy, are warranted.

A second model-associated weakness has to do with the validity of the protocol as a model of cardiac arrest. No model can duplicate exactly a clinical condition, though every attempt should be made to mirror the disease of interest so that general conclusions can be made. I have begun to address this issue through the development of a new model that incorporates a period of low flow ischemia following no flow ischemia. Hopefully this will more closely emulate a typical cardiac arrest by including the period of CPR that usually occurs during resuscitation. It will also provide a model that allows study of ROS generation in a more clinically relevant condition. The data presented show that a period
of low flow perfusion can alter the metabolic and functional consequences of I/R. The most unexpected finding was that by decreasing the no flow ischemia time with low flow below some as yet undetermined threshold actually impairs ATP recovery during reperfusion.

A further variance in our model from a typical cardiac arrest is the lack of precipitating and ongoing cardiac arrythmias before and during ischemia. Most cases of cardiac arrest are associated with ventricular fibrillation (VF) (8). The electrical activity of the hearts in the current model was not measured, though they did not routinely demonstrate the quivering that is indicative of VF. Lack of VF during ischemia may have profound effects on post-ischemic outcomes, as VF is associated with increased energy consumption (7). Development of a model in which ischemia is preceded by VF may provide better insight into the mechanisms of clinical cardiac arrest.

Despite the problems just mentioned, the results presented are quite provocative, especially in that they highlight a potentially important role for ROS generated during ischemia. This is further evidence that, while most previous research has focused on the large burst of ROS at reperfusion, low levels of oxidants act as cell signals (175) and may actually be critically important for cell survival.

The results suggest several future directions. The most obvious next step would be to repeat some of the experiments in a model in which AOX treatment results in improvement on post-ischemic function. This would allow conclusions to be drawn regarding the importance of the findings in the development of contractile dysfunction. The use of transgenic models that overexpress particular aspects of endogenous AOXs would allow a more detailed localization of the ROS generation, and determination of the
particular ROS involved. Furthermore, it would be useful to show that AOX treatment decreases oxidant levels, and that the results can be reproduced through application of oxidants, through exogenous generators, in the absence of ischemia and reperfusion.

The next set of experiments would be directed at determining the mechanisms by which ROS generated during ischemia exert their effects. Experiments pertaining to high-energy phosphate recovery would look at processes in which ROS have been shown in other models to either increase contractile efficiency, for example, by activating PKC (177), or to stimulate energy production, such as by activating glycolysis (92, 106, 157).

In terms of Tn I proteolysis, determining the mechanism by which oxidants regulate degradation would include identification of the protease responsible for oxidant sensitivity, and to determine any structural changes that may increase Tn I susceptibility to proteases, such as exposure of hydrophobic residues. It would be interesting to see if generating mutant proteins lacking oxidizable residues could alter degradation.

The novel model of cardiac arrest presented offers a plethora of new studies. These include determination of the threshold of flow that is necessary to improve energetic recovery. Low flow may also alter the burst of ROS that is seen upon reperfusion, and attenuate Ca$^{2+}$ influxes through a damping of the pH swing seen at reperfusion. Finally the effect, if any, of an intervening period of low flow on any of the factors thought to be ultimately responsible for post-ischemic dysfunction, such as decreased Ca$^{2+}$ sensitivity or Tn I degradation, is currently unknown.
Unfortunately, the inability to attenuate skeletal muscle fatigue with antioxidants and further difficulties with initial energetic work precluded drawing any conclusions regarding ROS mediated regulation of skeletal muscle contractile function. However, a novel model of fatigue was developed and verified.

The role of ROS is far more complex than that of the "chemical vandals", randomly damaging anything in sight, they were once thought to be. This dissertation provides further evidence, in an important clinical model, of a role for ROS in regulation of cell function.


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