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UMI
REACTIVE METABOLITES OF OXYGEN AND NITROGEN
INFLUENCE RAT DIAPHRAGM MUSCLE FUNCTION DURING
HYPOXIA

DISSertation
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University
By
Priya Mohanraj, M.D., M.S.

*****
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1998

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ABSTRACT

During hypoxia, a condition called reductive stress, characterized by excess reducing equivalents in the cell, can paradoxically lead to enhanced formation of reactive species of oxygen and nitrogen and may lead to conditions of oxidative stress. The hypothesis tested in this thesis was that the decline in function seen during hypoxia is in part caused by reactive species of oxygen. If so, then treatment with antioxidants should inhibit the fall in muscle function during hypoxia. Similarly, if the fall in muscle function during hypoxia is due to reactive species of nitrogen, then reduction in endogenous nitric oxide (NO) production should protect muscle function and conversely, addition of exogenous NO should further inhibit muscle function during hypoxia. Finally, if hypoxia triggers formation of reactive species of oxygen or nitrogen, it is possible that this results in conditions of oxidative stress characterized by a shift in glutathione redox status or increased lipid peroxidation products. These hypotheses were tested on untreated (control) rat diaphragm muscle strips incubated in tissue baths and tissues treated with antioxidants such as N-acetyl-L-cysteine, dimethylsulfoxide, superoxide dismutase and Tiron or, modulators of nitric oxide production such as nitric oxide synthase inhibitor (L-NAME), NO donor (DETA/NO) or guanylate cyclase inhibitor (ODQ). Force-frequency relationships were studied under an initial phase (95%O₂-5%CO₂), after a 30 minute hypoxic phase (95%N₂-5%CO₂), and 30 minutes after
a recovery phase. The results showed significant protection of muscle function during hypoxia in muscles treated with antioxidants, NO donors and nitric oxide synthase inhibitors. ODQ did not affect muscle function during hypoxia, suggesting that endogenous NO functions via cGMP-independent pathways. Upon reoxygenation, there was a significant recovery of muscle function in antioxidant-treated tissues, while modulators of nitric oxide had minimal effects on recovery. There was however, no clear evidence of oxidative stress as determined by an unaltered glutathione redox status and lack of significant changes in lipid peroxidation products. In conclusion, in this study of hypoxic diaphragm, while oxidant generation may be large enough to cause significant decline in muscle function, it is not of sufficient magnitude to cause significant, irreversible oxidative stress to the muscle.
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CHAPTER 1

INTRODUCTION

Free radicals are highly reactive chemical species possessing a free unpaired electron. They were first described by Gomberg in 1900. However, it was not until 1966 when the tissue injuring properties of free radicals were recognized by Slater who proposed that the hepatotoxicity of carbon tetrachloride was due to a free radical reaction (16). It is now known that free radicals can cause widespread damage by oxidizing lipids, proteins, enzymes as well as the DNA of a cell (16). The term reactive oxygen species (ROS) or reactive oxygen metabolites (ROM) or active oxygen (AO) encompasses a broad category of reactive chemical species including oxygen free radicals such as OH, O$_2^-$ etc., as well as other molecules with oxidizing properties such as H$_2$O$_2$, HOONO, HOCl. In addition to ROS, several reactive species of nitrogen or reactive nitrogen species (RNS) have been described, which ultimately originate from the free radical, nitric oxide (NO) (40). This section will briefly discuss the ROS, RNS, the potential role of reactive species in hypoxia, and muscle fatigue. Table 1 illustrates some of the known ROS and RNS (21).
### Reactive species of oxygen (ROS) | Reactive nitrogen species (RNS)
---|---
Superoxide \((O_2^-)\) | Nitric oxide \((NO)\)
Hydroxyl \((\cdot OH)\) | Nitrogen dioxide \((NO_2^-)\)
Peroxyl \((LO_2^-)\) | Nitrous acid \((HNO_2)\)
Alkoxy (LO·) | Dinitrogen trioxide \((N_2O_3)\)
Hydroperoxyl \((HO_2^+)\) | Dinitrogen trioxide \((N_2O_4)\)
Hydrogen peroxide \((H_2O_2)\) | Peroxynitrite \((ONOO^-)\)
Hypochlorous acid \((HOCI)\) | Alkyl peroxynitrites \((LOONO)\)
Ozone \((O_3)\) |  
Singlet oxygen |  
Lipid peroxides | 

Table 1. Reactive species of oxygen and nitrogen
REACTIVE OXYGEN SPECIES

ROS are produced in the cell either as a byproduct of metabolism, or deliberately, such as the ROS produced by phagocytes (16). Some ROS are free radicals, (e.g. $O_2^-$ or OH), defined by an unpaired electron, whereas other ROS such as $H_2O_2$ are not because they have no free electron. Under normal biological conditions oxygen causes oxidation; that is, it removes electrons from other molecules and in the process becomes reduced. The resulting break-up of an electron pair leads to free radical formation (16). Thus, depending upon the number of electrons transferred, a single electron reduction product of oxygen results in superoxide formation, transferring of two electrons results in $H_2O_2$ formation and transfer of a third electron to $H_2O_2$ by ferrous iron results in the lysis of the O-O bond, thus giving rise to hydroxyl radical (OH), which is a highly potent oxidant (62). The latter reaction is also known as the "Haber-Weiss reaction":

$$Fe^{2+} \rightarrow O_2^- + H_2O_2 \rightarrow OH + OH^+ + O_2 \quad \text{Equation 1}$$

The above reaction may be triggered to a greater extent in conditions of hypoxia and/or acidosis, where iron and other metals can become more available (80). Under normal conditions, iron is stored in various storage sites within the cell and is not freely available. However, during ischemia or acidosis, the cell loses its ability to maintain pH homeostasis and can result in liberation of free iron from storage sites (30,74).
Sources of ROS

There are several known cellular sources of free radical generation (34). Free radicals can be produced by auto-oxidation of low molecular weight species such as thiols, hydroquinones and flavins (16). They may also be produced by certain enzymes such as xanthine oxidase, cyclo-oxygenase, cytochrome P450 reductase and mitochondrial NADH dehydrogenase (16). The cell uses these enzymes to oxidize other compounds and, in the process, reduces oxygen to O_2^-\textsuperscript{•}. The mitochondria generate free radicals even during its normal respiration, where about 2% of the electron flow is believed to be shuttled towards reduction of O_2 to form superoxide anion (28). Normally powerful antioxidant systems within mitochondria quench free radical production and prevent widespread damage. Of particular importance to this thesis is the fact that mitochondrial production of free radicals may increase in conditions of "reductive stress" which is characterized by a build-up of reducing equivalents such as NADH/NAD\textsuperscript{+} in the cell during conditions of tissue hypoxia (87). This will be discussed in detail later. The peroxisomes, which have high levels of oxidases, are another source of free radicals which are used to metabolize or detoxify unwanted compounds (34). Free radicals can also be produced by the endoplasmic reticulum and the nuclear membrane both of which contain cytochrome P450 and cytochrome b5, which are enzymes that can oxidize unsaturated fatty acids while reducing dioxygen. The endoplasmic reticulum generates free radicals to assist in the folding of proteins by oxidizing sulphydryl groups (28,34). The plasma membrane is also believed to be a site of normal ROS formation by membrane bound oxidases. The function of these, or how ubiquitous they are, in different tissues is not well known (19,28,61). All membranes can undergo chain auto-oxidation
reactions thus involving a large number of free radicals in the process (16,28,34). In addition, cell membranes are associated with lipid oxidizing enzymes such as lipoxygenase and cyclooxygenase which can generate free radicals during their normal pathway of action (28). During inflammation, the *cells of inflammation* such as the phagocytes, neutrophils and macrophages are all capable of generating large amounts of reactive oxygen species as part of their microbicidal killing mechanisms. Finally, in a more global setting, several *environmental factors* such as hypoxia, ischemia-reperfusion, radiation, inflammation and hyperthermia can enhance free radical production by influencing any one or more of the above mentioned sources (28).

**Biological effects of ROS**

ROS have been implicated in the pathogenesis of several diseases and pathological conditions such as, autoimmune diseases, atherosclerosis, Alzheimer's disease, carcinogenesis, cigarette-smoke effects, emphysema, glomerulonephritis, as well as in normal aging (20). The effects of ROS may largely be attributed to their oxidizing reactions on lipids, proteins, carbohydrates, and nucleic acid in cells (7,16).

**Lipid peroxidation reactions**

The oxidation of poly-unsaturated fatty acids is known as lipid peroxidation (7). This can result in membrane lipid cross-linking, changes in cell and organelle permeability, as well
as self-perpetuating oxidation reactions via peroxyl radicals (16,28). The resulting alteration of membrane permeability can affect ionic balance within the cell, thereby influencing cellular homeostasis (7).

Protein and nucleic acid oxidation

Proteins and nucleic acids are less susceptible to free radical attack, as compared to lipids because they may not result in destructive self-perpetuating chain-reactions as the latter (16). Free radicals may attack critical sulfhydryl groups on proteins or active sites of enzymes, thereby inactivating enzyme-systems (7). For instance, O$_2^·$ radical has been shown to inactivate endogenous antioxidant enzymes such as catalase and glutathione peroxidase (36). Like proteins, oxidation reactions of nucleic acids may not be self-propagating (16). Oxidation of DNA can compromise cell viability or result in mutations, which upon accumulation over a lifetime have been shown to induce certain diseases including carcinogenesis (16).

Second messenger systems

Reactive species can also activate second messenger systems. For instance, one of the proposed mechanisms of biological effects of NO is via cGMP, from activation of the enzyme, guanylate cyclase by NO (57,59,88). Similarly, O$_2^·$ has been shown to promote fibroblast proliferation and H$_2$O$_2$ has been shown to activate transcription factors such as NFκB (97). In addition, several other genes important for cell growth and differentiation
such as, c-fos, c-myc, c-jun and β-actin have been shown to be induced by ROS (97). It has been proposed that unregulated activation of such genes can also result in carcinogenesis (97).

**Antioxidant mechanisms of the body**

Even under physiological conditions, oxidative species are continuously formed within the body which are scavenged by several endogenous antioxidants. These include specific intracellular enzymatic defenses such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as other small molecule antioxidants such as tocopherols, ascorbate, glutathione, ubiquinol-10, and urate (14). Although these antioxidant systems of the cell protect against oxidants, they can be easily overwhelmed under conditions of oxidative stress, where there is an excess production of oxygen free radicals.

**Superoxide dismutase**

SOD was first discovered in 1969 and is a $O_2^-$ scavenger. There are two forms of SOD; copper-zinc SOD, found mainly in the cytosol and perhaps also in the nucleus, and manganese-SOD found in mitochondria (40). SOD eliminates superoxide anion $(O_2^-)$ by catalyzing its dismutation to $H_2O_2$ and $O_2$.

$$SOD \quad 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{Equation 2}$$

The enzymatic degradation of $O_2^-$ is approximately $10^4$ times faster than its spontaneous degradation (40). Radak et. al. demonstrated an increased SOD activity in rat skeletal muscle.
following intense exercise. Furthermore, they found decreased lipid peroxidation products following exercise, in animals treated with exogenous SOD (72).

**Catalase**

Catalase is a 240,000 D, heme containing enzyme which catalyzes the breakdown of H$_2$O$_2$ into water (H$_2$O) and molecular oxygen.

$$\text{Catalase}$$

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \quad \text{Equation 3}$$

Catalase is present in all tissues, although the highest levels are present in liver and erythrocytes. A predominant intracellular location is within the peroxisomes, where a large amount of H$_2$O$_2$ is produced, although other locations of catalase are also known (24,40).

**Glutathione peroxidase**

Glutathione peroxidase (GPx) is also a metalloenzyme containing selenium (molecular weight of 85,000), which helps to eliminate H$_2$O$_2$. It catalyzes the breakdown of H$_2$O$_2$ by causing simultaneous oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) (40).

$$\text{glutathione peroxidase}$$

$$2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \quad \text{Equation 4}$$

GSSG, in turn, recycles back to GSH in a reaction catalyzed by glutathione reductase

$$\text{glutathione reductase}$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \quad \text{Equation 5}$$

Glutathione (γ-glutamyl-cysteinyl-glycine) is an abundantly present low molecular weight tripeptide in the body (25). It is present in high concentration intracellularly (0.5-10 mM) and
relatively lower concentration extracellularly (in \( \mu M \)) (63). This system is composed of two tightly coupled enzymes, namely, glutathione peroxidase and glutathione reductase which regulate GSH and GSSG levels in the cell (25). It is one of the most important cellular antioxidant systems in the body by virtue of its thiol group. In addition, it is a free radical scavenger (25, 64).

Glutathione is synthesized via the \( \gamma \)-glutamyl cycle by the enzyme glutathione synthetase (25). It is believed that the transportation of amino-acids required for glutathione synthesis is well regulated by a membrane bound \( \gamma \)-glutamyl transpeptidase (63, 65). A lowering of GSH to 20-30\% of its physiological levels may result in impaired cellular defense, cell injury and even cell death (78). Under normal conditions, GSSG is easily reduced back to GSH via the reductase enzyme and thus can replete GSH levels. However, under conditions of oxidative stress, it may be possible for the excess GSSG levels to overwhelm glutathione reductase capacities (78). Consequently, a build-up of intracellular GSSG can occur which is toxic to the cell and can be exported out of the cell (25, 78). Increased oxidized glutathione has also been demonstrated in several studies on skeletal muscle under conditions of intense muscle activity such as in resistive loading of the diaphragm and exercise of limb muscles (2, 18, 51, 85).

Non-enzymatic antioxidant systems

There are several non-enzymatic antioxidant systems of the body with different mechanisms of action, Table 2 (40). They are non-enzymatic in nature and either scavenge or decrease/inhibit production of toxic reactive oxygen species. In general, unlike most
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<td>Ascorbic acid</td>
<td>Water soluble free radical scavenger</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Water soluble free radical scavenger</td>
</tr>
<tr>
<td>Sulfhydryl groups</td>
<td>Water soluble free radical scavenger</td>
</tr>
<tr>
<td>Glucose</td>
<td>Water soluble hydroxyl radical scavenger</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Water soluble hydrogen peroxide scavenger</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>Lipid soluble free radical scavenger</td>
</tr>
<tr>
<td>β-carotene</td>
<td>Lipid soluble free radical scavenger</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Water soluble free radical scavenger</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Plasma protein binding iron</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Plasma protein binding copper</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Plasma protein binding hemoglobin</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>Plasma protein binding free heme</td>
</tr>
</tbody>
</table>

Table 2. Non-enzymatic antioxidant systems of the body
enzymatic antioxidants which are confined intracellularly, non-enzymatic antioxidants can also be present in extracellular fluids such as plasma and lymph (40).

**REACTIVE NITROGEN SPECIES**

The presence of nitrogen oxides in mammalian cells was first suggested by Mitchell et al. in 1916 (88). However, it was in 1980 when endothelium derived relaxing factor (EDRF) was identified, and in 1985 it was known that EDRF was actually nitric oxide (NO), responsible for the smooth muscle relaxation produced by acetylcholine in preparations with intact endothelium (57). It is now known that NO promotes relaxation through the cGMP pathway, Fig. 1 (57,88). NO mediates a wide variety of physiological actions including vasodilation, neurotransmission, inhibition of platelet aggregation, regulation of basal blood pressure and macrophage-mediated cytotoxicity (40,57).

**Sources of NO**

Nitric oxide is produced from oxidation of L-arginine by the enzyme NO synthase (NOS). Three different isoforms of NOS have been identified so far. These include calcium-dependent constitutive NOS (endothelial cell NOS/ecNOS and neuronal NOS/nNOS) and inducible NOS (iNOS), which is usually induced by bacterial products, bacterial invasion and
Figure 1. Biosynthesis of Nitric oxide
inflammatory processes (88). Diaphragm muscle fibers express all three types of NOS (37,49). Interestingly, Gath et. al. showed the presence of iNOS expression even in normal (specific pathogen-free) resting guinea pig diaphragm (37).

In skeletal muscle cells, NOS is found to be specifically located within the cell. It has been shown that ecNOS and iNOS are located within the sarcoplasm, while nNOS is located close to sarcolemma (37,59,60). It is unclear if presence of NOS in skeletal muscle may be fiber-type specific (49). Kobzik et al. showed expression of nNOS in type II (fast twitch, glycolytic) fibers (59), while ecNOS was expressed predominantly in oxidative fibers which are rich in mitochondria (60). Furthermore, some fibers were found to co-express ec-NOS and nNOS (60). However, in their studies Hussain et. al. did not find any correlation between fiber type and muscle NOS activity (49). In addition, these investigators demonstrated significant intra-species and inter-species differences in skeletal muscle NOS activity.

**Biological targets of RNS**

NO is a ubiquitous, rapidly diffusible intercellular messenger containing an unpaired electron. In biological systems, the highly reactive properties of NO are attributed, not only to the free radical nature of NO, but also because of its capacity to react with certain other molecules, such as other free radicals and transition metals (9). NO can behave both as a pro-oxidant or antioxidant, depending upon the relative concentration of NO and other reactive species that are present (35). Interaction of nitrogen oxides with metals and thiols
which are located on several key enzymes, receptors, ion channels and transcription factors is considered to be a predominant mechanism by which NO participates in redox signaling and activation of second messenger systems (81).

**Pro-oxidant effects of NO**

NO and RNS have been shown to be toxic to the cell by their direct interaction with iron-sulfur proteins, inhibition of ribonucleotide reductase and other enzyme systems, nitrosylation of thiols as well as inhibition of protein synthesis (33,82). NO can also exert indirect toxic effects via peroxynitrite, which is formed as a result of its combination with O$_2^-$ (71).

\[
O_2^- + NO \rightarrow OONO \quad \text{(peroxynitrite)} \quad \text{Equation 6}
\]

Peroxynitrite is a potent oxidant which has been shown to oxidize thiols, sulfides, lipids and deoxyribose (71).

At physiological pH, peroxynitrite behaves as a powerful oxidant by three pathways, Figure 2 (8):

a) by directly oxidizing sulfhydryl groups.

b) by decomposition to a ‘hydroxyl-nitrogen dioxide’ (OH$^-$· NO$_2^-$) intermediate. This hydroxyl radical-like species is highly reactive and easily oxidizes any biological molecule in its vicinity and,

c) by interaction with metals to form nitronium ion (NO$_2^+$), a potent nitrating species (8).
ONOO$^-$ + R-SH $\rightarrow$ R-S$_{ox}$

ONOO$^-$ + H$^+$ $\rightarrow$ "OH .... ·NO$_2$"

ONOO$^-$ + SOD/Metals $\rightarrow$ NO$_2^+$ + ·OH

Figure 2. Oxidative pathways of peroxynitrite
**Antioxidant effects of NO**

Increasing evidence suggests that NO also protects cells from cytotoxicity by reactive oxygen species (67,77,94,96). It is believed that NO may play a protective role by preventing formation of oxidants, by interacting with metals and preventing their participation in other redox reactions, scavenging the radicals and oxidants formed, and/or by terminating lipid peroxidation by interaction with lipid radicals (77,95). Kanner et al. demonstrated that NO functions as an antioxidant by preventing hydrogen peroxide-mediated cytotoxic effects on myoglobin by reducing the activated ferryl myoglobin to metmyoglobin (53). Furthermore, in a previous study they showed that NO prevents lipoxygenase and cyclooxygenase mediated lipid peroxidation (53).

The antioxidant effect of NO in the prevention of lipid peroxidation has also been demonstrated in other studies (41,47), where NO was shown to rapidly scavenge lipid peroxyl radicals (47). Recently, White et al. showed that NO can also increase levels of the endogenous antioxidant, glutathione in rat lung fibroblasts exposed to NO donors for 6-12 hours, perhaps by up regulating glutathione biosynthesis (93).

Therefore, NO seems to participate in competing reactions in the cell, some of which promote oxidation reactions and others of which block oxidation reactions. The particular role it plays depends entirely on the target molecules, the relative concentration of NO and other factors such as level of $O_2^-$ or other local tissue conditions.
Effect of NO on skeletal muscle

NO has been suggested to play an important role in skeletal muscle function (15, 50, 59). As discussed earlier, skeletal muscle expresses all three forms of nitric oxide synthase. Under normoxic conditions, NO donors have been shown to depress contractile force, while NOS inhibitors improved contractile function (59). It is believed that NO influences skeletal muscle contractile function either by directly affecting excitation-contraction coupling, or by regulating metabolic pathways by its action on critical enzymes (such as glyceraldehyde 3-phosphate dehydrogenase), proteins, or via signal transduction (1, 6, 29, 75, 83, 89).

NO has been shown to bind directly to the calcium release channel (ryanodine receptor) and also prevent oxidation of the ryanodine receptor by oxidants, by inhibiting cross-linking between the receptor subunits (1, 83). Similar reports of the influence of NO on intracellular calcium have shown in cardiac cells (isolated human atrial myocytes), that treatment with the peroxynitrite donor, SIN-1 results in an increase in the cardiac calcium current \( (I_{Ca}) \) possibly by molecular interaction with the Ca\(^{2+} \) channel protein (58). Finally, several studies have reported that NO can facilitate glucose uptake in both resting as well as contracting skeletal muscle (5, 6, 29, 76, 101). This may be of physiological significance for this thesis, because an energy-deficient hypoxic muscle must be dependent on glycolysis for ATP production, resulting in a high degree of glucose turnover.
HYPOXIA AND REACTIVE METABOLITES OF OXYGEN AND NITROGEN

Several studies have demonstrated that hypoxia alone, without subsequent reoxygenation or reperfusion, can result in the production of oxidants in different tissues (66,69,70,87). There are a number of potential sources for reactive oxygen species in hypoxia. One source may be the mitochondria (23,55,56,69,70). Even under normal aerobic conditions, about 2% of the total mitochondrial oxygen consumption is shuttled toward the formation of $\mathbf{O}_2^\cdot$. This is believed to be generated at complex I and complex III of the electron transport chain (23). During hypoxia, less $\mathbf{O}_2$ is available to be reduced to $\mathbf{H}_2\mathbf{O}$ at cytochrome oxidase, resulting in an accumulation of reducing equivalents (starting with increased NADH) within the mitochondrial respiratory sequence, a condition sometimes referred to as reductive stress (55). This can result in the formation of ROS by the auto-oxidation of one or more mitochondrial complexes such as the ubiquinone-ubiquinol redox couple (4). Rao and Mueller showed that even at a tissue $\mathbf{P}_\mathbf{O}_\mathbf{2}$ of 1 mm Hg (normal, 35 mm Hg) there can be a significant production of free radicals (73).

Other possible sources of ROS during hypoxia include activation of certain enzymatic pathways such as xanthine oxidase and cyclooxygenase. During hypoxia an insufficient supply of ATP can result in the malfunction of ATP-dependent calcium pumps of the cell (42). The excess calcium ions stimulate the transformation of xanthine dehydrogenase to xanthine oxidase by the action of calcium-activated proteases. Xanthine oxidase catalyzes the breakdown of hypoxanthine (formed as a catabolic product of adenine nucleotides in increased amounts during hypoxia) into xanthine and uric acid, releasing $\mathbf{O}_2^\cdot$ (13,42). Several
studies (13,39,46) have shown an increased activation of the xanthine oxidase pathway during hypoxia and/or on subsequent reoxygenation in a variety of tissues. Reactive oxygen species may also be formed by cytosolic enzymes such as cyclooxygenase. Under hypoxic conditions there is an increased activation of the enzyme phospholipase A₂ which releases arachidonic acid from phospholipids. Cyclooxygenase reacts with this accumulated fatty acid, simultaneously generating singlet oxygen and hydroxyl radical (OH) (15,17).

Finally, hypoxia can also cause increased NO production. There is evidence to suggest increased production of NO during both acute and chronic hypoxia (44,98-100). Studies on endothelial cells subjected to an acute hypoxic environment suggest that increased production of NO was stimulated by elevated levels of cytosolic calcium (44) or, by increased activity of cNOS occurring during hypoxia (98). This simultaneous presence of reactive oxygen intermediates and NO may have several important implications on biological systems since they both can act as cell signaling agents, as modulators of enzyme activity and ion channel activity (75).

Furthermore, both ROS and NO can affect muscle contraction by producing opposing effects on release of calcium from sarcoplasmic reticulum (75). Oxidation reactions by reactive oxygen species of sulfhydryl groups on sarcoplasmic reticulum (SR) can cause inhibition of the SR-pump, thereby resulting in increased intracellular calcium due to decreased reuptake. On the contrary, NO produced by different nitric oxide synthases, can inhibit calcium release by promoting reuptake via cGMP-dependent activation, by directly interacting with ROS and inhibiting them, or both (75).
EFFECT OF HYPOXIA ON CELLULAR MECHANISMS

Energy requirements of the cell during hypoxia

Hypoxia is a condition characterized by low tissue oxygen concentration. Under normal conditions, about 60% of the aerobically produced ATP comes from the metabolism of fatty acids, whereas glucose and lactate metabolism account for only 11% and 29% of ATP production respectively (42). However, during hypoxia there is an inhibition of the carnitine-acylcarnitine translocase (enzyme involved in fatty acid metabolism). This leads to an impairment in the transportation of long chain fatty acids into the mitochondria. As a result, during hypoxia there is a preferential utilization of glucose as a metabolic substrate. This is called the "Pasteur effect". Hypoxia also causes an inhibition of peptide chain elongation thus inhibiting protein synthesis. This results in an increased accumulation of amino acids within the cells (42,90).

As the oxygen supply decreases, the rate of ATP hydrolysis may exceed the rate of ATP production, resulting in an increased accumulation of ADP, P_i, and H^+ ions (38). These metabolites act as feedback signals and promote anaerobic production of ATP by glycolysis, creatine kinase and adenylate kinase reactions (42,90).

Effect of hypoxia on redox balance of the cell

One of the primary mechanisms for free radical scavenging in the cell is the glutathione redox system (64). As described earlier, this system is composed of two tightly coupled enzymes, namely, glutathione peroxidase and glutathione reductase. These enzymes
are found mainly in the cytosol, although some glutathione redox activity is also found in the mitochondria (87). Glutathione reductase requires NADPH as a cofactor. NADPH is produced by NADP+ specific dehydrogenases and from trans-hydrogenation in the mitochondria. The hexose-monophosphate shunt is one of the major sources of NADPH (7). This pathway is regulated by the enzyme glucose-6-phosphate dehydrogenase (G6PD), which is inhibited by NADPH at physiological concentrations. G6PD is activated when NADPH/NADP+ and GSH/GSSG ratios are decreased and therefore G6PD enhances the recovery of NADPH and thereby, GSH pools under oxidizing conditions (87). The glutathione redox system is a reliable indicator of oxidative stress in the cell (7). Under conditions of oxidative stress, there is a loss of total intracellular glutathione (GSH) associated with an increase in oxidized glutathione (GSSG) (7). Excess GSSG is cytotoxic to the cell because it leads to protein sulfhydryl-GSSG exchange which can be detrimental to protein function (48). Increased GSSG/GSH ratio in the rat diaphragm has been shown by Anzueto et. al. during resistive breathing protocol, which is characterized by oxidative stress (2,85).

Similarly, several studies in hypoxia have demonstrated that hypoxia alone, without subsequent reoxygenation or reperfusion, can result in production of oxidants in some tissues (69,70,87). This oxidant stress can result in a loss of total intracellular GSH associated with an increase in GSSG, resulting in high GSSG/GSH ratio (54,70).
Cellular ionic changes during hypoxia

Studies on the heart by Park et al. showed that maximum oxidant stress occurs in response to hypoxia and further reoxygenation did not produce a marked difference in the oxidative damage (69). Increased susceptibility to cellular damage during hypoxia may not only be due to generation of reactive oxygen species but could also be due to increased intracellular acidosis, loss of adenine nucleotides from the cell or effects of elevated intracellular calcium levels (42). Increased intracellular calcium could occur due to inhibition of Na⁺-Ca²⁺ exchanger (most active in heart and brain cells) and of the calcium pump during hypoxia (42, 68). It has been proposed that the fall in muscle tension (muscle dysfunction) seen during repetitive contractions of skeletal muscle could be due to an inhibition of calcium release, decreased calcium sensitivity of the contractile proteins, inhibition of T-tubular conduction of the action potential and/or due to inhibition of calcium pumping ability of the sarcoplasmic reticulum (31, 91). The excess calcium ions activate proteases which can destroy the sarcolemma and cytoskeleton. Hypoxia can also cause breakdown of membrane phospholipids by several mechanisms (68). This may occur due to the action of calcium-activated phospholipase, by an energy deficit which prevents phospholipid re-synthesis or due to cell membrane rupture caused by an alteration of the shape of the cells (under extreme conditions of hypoxia) (68, 91).
SKELETAL MUSCLE FATIGUE

When skeletal muscles undergo repetitive contractions over a period of time, they undergo a decline in performance, usually seen in the form of decreased twitch tension and decreased maximum tetanic tension. This phenomenon is called muscle fatigue and is defined as the reversible time-dependent impairment in the capacity to generate force, in response to sustained stimulation (10). The mechanism of fatigue in a muscle may be closely related to the mechanism of dysfunction in a hypoxic muscle. This may be by virtue of the proposed role of oxidant stress in both conditions (70,84), as well as by several common intracellular metabolic changes in the two muscles (38). The latter include a rise in inorganic phosphate, decline in pH, a fall in phosphocreatine levels and changes in the concentrations of adenine nucleotides, all of which can directly depress the contractile apparatus, causing a decline in muscle function (38). This section will briefly discuss the known mechanisms of muscle fatigue.

Normal and altered mechanisms in the muscle

Similar to other skeletal muscles, diaphragm has also been shown to undergo fatigue in response to severe work loads, resulting in respiratory failure (3). The exact cellular mechanism of muscle fatigue is still unknown. Intense muscle stimulation, as occurs during
respiratory failure, may trigger fatigue due to a failure in any of the possible sites involved in muscle activation (92). These include:

i) failure of central command

ii) inhibition of conduction of action potential to neuromuscular junction

iii) failure at the neuromuscular junction

iv) loss of action potential activation at end-plate area

v) disruption of propagation of action potential along sarcolemma and/or along t-tubules

vi) decreased release of Ca\(^{2+}\) from sarcoplasmic reticulum

vii) failure of uncovering of the active sites on actin

viii) decreased cross-bridge cycling

ix) decreased calcium reuptake by sarcoplasmic reticulum.

**Cellular mechanisms of muscle fatigue**

**Metabolic factors in fatigue**

Under normal conditions, most of the ATP supply to the muscle comes from the aerobic metabolism of fatty acids (42,90). However, during intense muscular activity there is increased utilization of ATP. The immediate source for the replenishment of ATP comes from the creatine phosphokinase reaction where phosphocreatine (PCr) is consumed with concomitant formation of creatine and P\(_i\)

\[
\text{PCr + ADP + H}^- \rightarrow \text{Cr + ATP} \quad \text{Equation 7}
\]

\[
\text{ATP + H}_2\text{O} \rightarrow \text{ADP + P}_i + \text{H}^- + \text{energy} \quad \text{Equation 8}
\]
Thus, with continued activity there is a decrease in PCr while there is increased accumulation of Pi and creatine. A further source of ATP comes from the activation of glycogenolysis and glycolysis (anaerobic pathways), which leads to the formation of lactate (92). In contrast to PCr, several studies have shown that the concentration of ATP shows only modest declines, rarely falling below 60-70% of pre-exercise values, even with extensive fatigue (32,92). Hence, it is generally believed that energy availability is not a limiting factor in the pathogenesis of muscle fatigue (43).

**Electrolyte changes during fatigue**

Most excitable cells, including skeletal muscle cells, undergo ionic shifts across the cell membrane with repeated activation (92).

**i) changes in sodium and potassium**

Studies on frog skeletal muscle fibers have shown that during intense fatigue of fast twitch fibers there is a significant decline in the intracellular potassium level ([K]_i), with a simultaneous increase in intracellular sodium ([Na]_i) levels (52). It is believed that potassium efflux and inhibition of Na⁺ - K⁺ pump may result in cell depolarization, decreased action potential amplitude or even complete inactivation, thus preventing the initiation of the excitation-contraction (E-C) coupling process. However, it has been shown that most cells depolarize only by 10-20 mV during fatigue and this change does not affect the propagation of action potentials down the T-tubules or subsequent steps in E-C coupling (32). Nevertheless, membrane potential changes have never been measured across T-tubule in fatigue.
ii) changes in calcium

In the skeletal muscle, calcium plays two primary regulatory roles, namely it is the intracellular messenger for excitation-contraction coupling and it activates cellular enzymes involved in energy production (86). Thus, calcium not only activates tension development but is also required for the regeneration of ATP in the cell. ATP is required by the skeletal muscle cell, not only for cross-bridge turnover but also for the maintenance of ion pumps, nuclear activity, etc. Calcium plays an important role in the pyruvate and fatty acid metabolism which form an important source of energy during normal aerobic metabolism of the cell. During energy deficient states, like intense activity or hypoxia, both fast and slow twitch muscle cells depend more on glycogenolysis and glycolytic pathways (anaerobic) for their energy requirements. Calcium is important in glycogenolysis by causing a calcium-dependent activation of the enzyme phosphorylase kinase. It is also important in glycolysis by facilitating compartmentation of the glycolytic enzymes into the terminal cisternae of the sarcoplasmic reticulum. Calcium may also be involved in glucose transport across the cell (86,90). In addition, calcium can cause activation of various calcium-dependent catabolic processes, which can result in cell injury. This occurs due to activation of various calcium-dependent degradative enzymes like phospholipases, proteases and endonucleases resulting in loss of membrane integrity, phospholipid degradation, DNA degradation, protein degradation, disruption of the cytoskeleton and ultimately cell death (42,68). Thus, intracellular calcium homeostasis is very important for normal cell function and viability.
Several studies have looked at the changes in calcium release during fatigue. Using Fura-2 as the calcium sensitive indicator, Westerblad et. al. (92) demonstrated an increase in intracellular calcium levels during early fatigue followed by a decline in the intracellular calcium levels in late fatigue. They proposed that the decline in tension may be due to several factors:

i) due to decreased calcium release from the sarcoplasmic reticulum: this may be due to loss in sensitivity of voltage sensors to changes in voltage or decreased open probability of calcium channels.

ii) decreased calcium sensitivity or increase in amount of calcium required to produce half-maximal tension (Ca_{50}). This may be due decrease in pH or increase in P_{i}, both of which increase the contraction threshold.

iii) reduced maximum calcium activated tension, which also may be due to acidosis or increase in P_{i}.

Free radicals and muscle fatigue

Recently, several studies on the diaphragm and other skeletal muscles have given evidence for the involvement of free radical species in muscle fatigue (2,3,11,12,22,27,45). One of the earliest pieces of evidence for free radical involvement during muscular exhaustion came from the studies of Davies et. al. (22) who showed increased free radical production using spin resonance techniques. Several studies on the diaphragm (3,11,12,27,79) have shown that free radicals are clearly involved in the diaphragmatic dysfunction seen during its repeated stimulation.
Anzueto et al. demonstrated an increased production of thiobarbituric acid-reactive substances (TBARS) and glutathione content in rat diaphragm, thus providing evidence of lipid peroxidation products due to free radical damage as well as simultaneous activation of the glutathione redox cycle (2). Previous studies in our laboratory by Diaz et. al. have demonstrated the generation of hydroxyl radical during diaphragmatic stimulation by using salicylate as the specific hydroxyl radical scavenger (27). In addition antioxidants such as, N-acetylcysteine (NAC), which is also structurally similar to glutathione, have been shown to attenuate diaphragmatic dysfunction (26,79).

This section on muscle fatigue is discussed in a greater detail at a later chapter.
LIST OF REFERENCES:


CHAPTER 2

ANTIOXIDANTS PROTECT RAT DIAPHRAGMATIC MUSCLE FUNCTION UNDER HYPOXIC CONDITIONS

ABSTRACT

In hypoxia, mitochondrial respiration is decreased, thereby leading to a buildup of reducing equivalents that cannot be transferred to oxygen at the cytochrome oxidase. This condition, called reductive stress, can paradoxically lead to enhanced formation of reactive oxygen species, or a decrease in the cell’s ability to defend against an oxidative stress. We hypothesized that antioxidants would protect tissues under conditions of hypoxia. Rat diaphragm strips were incubated in tissue baths containing one of four antioxidants, N-acetylcysteine, dimethyl sulfoxide, superoxide dismutase or Tiron®. The strips were directly stimulated in an electrical field. Force-frequency relationships were studied under baseline oxygenation (95%O₂-5%CO₂), after 30 minutes of hypoxia (95%N₂-5%CO₂), and 30 minutes after reoxygenation. In all tissues, antioxidants markedly attenuated the loss of contractile function during hypoxia ($P < 0.01$) and also significantly improved recovery on reoxygenation ($P < 0.05$). In conclusion, both intracellular and extracellular antioxidants improve skeletal...
muscle contractile function in hypoxia and facilitate recovery during reoxygenation, in an in vitro system. Their strong influence during hypoxic exposure suggests that they can be as effective in protecting cell function in a reducing environment as they have been shown to be protective in oxidizing environments.

INTRODUCTION

During respiratory failure and associated cardiovascular collapse and resuscitation, conditions are appropriate for local tissue hypoxia and ischemia-reperfusion to occur. Ischemia-reperfusion has been shown to be associated with the production of reactive oxygen species (ROS) in different organ systems (16,28,34). It is generally believed that they are primarily produced during the reperfusion period, when high O₂ concentrations promote the formation of superoxide (O₂⁻), through one electron reduction of oxygen. However, several studies have demonstrated that hypoxia alone, without subsequent reoxygenation or reperfusion, can result in the production of oxidants in some tissues (26,27,38). For example, Park et al. (27) have shown that in the heart, hypoxia results in both oxidant formation and oxidative stress which are not exacerbated further by subsequent reoxygenation. The mechanism responsible for oxidant production in hypoxia is not well understood and has not previously been demonstrated in skeletal muscle. The influence of hypoxia on oxidant production may be clinically important, because the working diaphragm can be exposed to
severe hypoxia during conditions of respiratory failure or cardiopulmonary arrest. Furthermore, a previous study in our laboratory has shown increased free radical generation in the diaphragm during conditions of severe respiratory failure (5).

Skeletal muscles have been shown to produce ROS both intracellularly and extracellularly at rest (31,33). Production appears to go up in response to intense, fatiguing exercise (5,12,31). Furthermore, during fatigue, force production and endurance are markedly preserved by treatment with a wide variety of antioxidants (31,33,36) and treatment with dithiothreitol, a potent reducing agent, improves recovery of muscle function following diaphragmatic fatigue (11). These previous experiments demonstrate that ROS or other oxidizing agents are generated in skeletal muscle and may contribute to the loss of force development in fatigue.

In this study, we explore the hypothesis that ROS, free radicals or other oxidizing agents are produced in hypoxic skeletal muscle and that these contribute to the loss of mechanical function associated with severe hypoxia. We reasoned that if oxidizing species are actively involved in contractile depression, then antioxidant treatment should preserve function, much like it is preserved during conditions of muscle fatigue. Alternatively, if oxidizing species are produced primarily during reoxygenation, then antioxidant treatment would not preserve function during hypoxia, but rather during the reoxygenation/recovery period.

Our results demonstrate that hypoxia causes significant muscle dysfunction which is markedly attenuated by antioxidant treatment. This effect appears to be independent of
whether the antioxidants work intracellularly or extracellularly. Furthermore, antioxidant treatment improves recovery upon reoxygenation.

METHODS

Experiments with antioxidants

Surgical procedure and muscle strip preparation

The experiments were conducted on diaphragm muscle strips taken from adult male Sprague Dawley rats (300-500 g). The animals (n=7) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg /kg), tracheotomized and mechanically ventilated. The phrenic nerve was severed and four diaphragm strips (approximately 0.5 cm) were dissected out with portions of rib and central tendon intact. The strips were divided into four groups. The control tissue was placed in a tissue bath containing ~125 ml of a physiological salt solution (in mEq/L: 21 NaHCO₃, 0.9 NaSO₄, 1.2 NaHPO₄, 0.9 MgCl₂, 2.25 CaCl₂, 5.9 KCl, and 109 NaCl and 2.07 g/L glucose). The experimental tissue was placed in a similar bath containing physiological salt solution with N-acetylcysteine (NAC, 18 mM), a commonly used antioxidant. The baths were maintained at 37°C and were continuously bubbled with 95%O₂-5%CO₂ during normoxia and 95%N₂-5%CO₂ during the hypoxic phase. Muscle function (twitch characteristics and stress-frequency relationships) was assessed at the end of each phase. To determine the kinetics of gas pressures in the tissue baths, the Po₂ in the baths was continuously monitored with a Clark electrode (BMS3 MK2
Blood Micro System, Radiometer, America Inc., Cleveland, OH). The $P_O_2$ generally dropped rapidly in the tissue baths, reaching <50 mmHg within 3 minutes. A minimum partial pressure, indistinguishable from zero, was reached within 15-20 minutes into the hypoxic period (Fig. 3).

The strips were mounted vertically with the central tendon positioned superiorly and attached to a force transducer which was connected to a micropositioner. D-tubocurarine, (10µM), a non-depolarizing neuromuscular blocking agent, was added to eliminate activation of intramuscular nerve branches. This drug binds with nicotinic receptors and prevents depolarization of muscle cell membrane, thus inhibiting contraction via acetylcholine (40). The diaphragm was stimulated to contract by placing it between two platinum electrodes and stimulating it directly in an electric field.

To confirm our findings, we repeated the experiments on three other antioxidants chosen because of their different modes of action, dimethyl sulfoxide (DMSO, 6.4 mM), superoxide dismutase (SOD, 500 U/ml), and 1,2-dihydroxybenzene-3,5-disulfonate (Tiron®, 10 mM) on diaphragmatic tissues (n = 5, in each group). The doses for the four antioxidants were selected based on previous in vitro studies in diaphragm and other tissues using these antioxidants (10,24,31). The surgical procedure was as described above. The experimental protocol was identical to NAC experiments except there were no normoxia time controls.
Figure 3. Washout of oxygen in the tissue bath during the hypoxic phase form a single representative experiment. It took approximately 20 minutes for the oxygen level to fall to less than 6 mmHg from an initial normoxic phase.
Figure 3.

Muscle function (Initial)

Muscle function (Hypoxia)

Muscle function (Recovery)
In these experiments one muscle strip served as control tissue, while each of the remaining three strips served as experimental tissue, treated with a different antioxidant, with all four tissues being studied simultaneously from the same animal.

**Stimulation paradigm**

After the strips were mounted in the tissue chamber, the maximum required stimulation current and the optimal length ($L_o$) were determined by adjusting the micropositioner between intermittent simulations. All stimulations were performed at $L_o$ and at supra-maximal current.

**Twitch characteristics**

Single twitches (0.5 Hz stimulation) were recorded and the peak twitch tension, time to peak tension, and half-relaxation time were calculated.

**Force-frequency relationships**

The muscle strips were tetanically stimulated at 20, 30, 40, 50, 60, 80 and 100 Hz with an interval of ~30 seconds between each tetanic stimulation; pulses were of 0.2 ms duration, with a train duration of 400 ms.

**Data Analysis**

At the end of the experiment, the length and wet weight of each muscle strip was obtained. For conversion of force to absolute stress, the cross-sectional area of each
diaphragm strip was determined by dividing the muscle mass (weight in g) by the product of muscle length in centimeters and muscle density (assumed to be 1.06 g/cm³) (8). All values are expressed as group means ± SE. Stress-frequency data for NAC experiments were analyzed using two-way repeated measures analysis of variance (ANOVA) with treatment and gas phase being independent variables and frequency being a categorical variable. Twitch data and individual tetanic force measurements for the other antioxidant experiments were also analyzed using a two-way ANOVA with treatment and gas phase being dependent variables. A P < 0.05 was considered to be statistically significant. Post-hoc comparisons were done using SAS JMP (SAS Institute Inc., Cary, NC) contrasts. Unless mentioned, P values in the text refer to post hoc contrasts.

RESULTS

MUSCLE FUNCTION DATA

Twitch Characteristics

Hypoxia caused significant reductions in peak tension (P = 0.002), half relaxation time (P = 0.001) and time to peak tension (P = 0.004), Fig. 4. In each case, these changes did not return to baseline during the reoxygenation phase. Although tension is expressed in % baseline, similar conclusions can be drawn from absolute stress measurements (not shown). Baseline measurements represent twitch characteristics taken in the initial/normoxic phase. During hypoxia, NAC significantly ameliorated the fall in peak twitch tension (P < 0.05) and inhibited the reduction in half relaxation time (P < 0.01), but had no significant effect on time
Figure 4. Twitch characteristics of the control untreated tissue (open bars) and NAC treated (hatched bars) tissues. (A) Peak twitch tension. (B) Half relaxation time. © Time to peak tension or contraction time. *P = change in the difference between untreated and NAC treated during the initial phase vs. hypoxic phase or, initial phase vs. recovery phase. For visual clarity, the graphical representation of tension is expressed as a % of baseline. Statistics were performed on non-normalized twitch stress (N/cm²). Remaining P values represent change in the same tissue during different phases, as noted.
Figure 4.
to peak tension. On reoxygenation, treatment with NAC did not significantly improve recovery of these variables.

**Stress-frequency relationships**

Treatment with NAC resulted in significantly lower initial (normoxic) stress measurements at low frequencies (20 Hz and 30 Hz), \( P < 0.05 \). However, the initial stress measurements at high frequencies (40 Hz - 100 Hz) were not affected by NAC treatment (Fig. 5A). This phenomenon has also been observed in previous studies on the diaphragm with NAC (23). In the hypoxic phase, there was a significant fall (ANOVA, \( P < 0.001 \)) in muscle stress (compared to initial stress), at all frequencies of stimulation. This was markedly attenuated (overall ANOVA, \( P < 0.001 \)) in tissues treated with NAC (Fig. 5B). Likewise, during reoxygenation, stress remained decreased at all frequencies of stimulation compared to initial stress (overall ANOVA, \( P < 0.001 \)). Stress during recovery was significantly improved (overall ANOVA, \( P < 0.01 \)) in tissues treated with NAC (Fig. 5C), Fig. 6.

The effects of other antioxidants (DMSO, SOD and Tiron®) on muscle function were similar to that of NAC. Peak twitch stress and the stress-frequency relationship during hypoxia and upon reoxygenation in muscles treated with these antioxidants were similar to NAC-treated tissues, Fig. 7, Fig. 8 and Fig. 9. Like NAC, Tiron® significantly lowered initial(normoxic) stress at low frequencies (30 Hz, Table 3). Also, like NAC, these antioxidants protected muscle function during hypoxia as well as upon reoxygenation. Although, the responses did not reach statistical significance at 30 Hz during hypoxia, they were significant at higher frequencies of stimulation (e.g., 100 Hz, Fig. 10, Table 3).
Figure 5  Comparison of the effect of Control and NAC-treated tissues on muscle function during each gas phase. Data are expressed as Means ± SE
Individual post hoc contrasts. *p < 0.05; effect from initial using repeated measures ANOVA, † † p < 0.001, ‡ ‡ p < 0.01
Figure 6. Summary of the effect of control and NAC-treated tissues on muscle function during the different gas phases. Circles = initial phase, Triangles = hypoxic phase, Squares = reoxygenation/recovery phase.
Figure 7. Effect of antioxidants on Peak Twitch Tension during hypoxia and recovery, compared to initial (100%) tension.
Figure 8. Effect of antioxidants on Twitch Time to Peak Tension.
Figure 9. Effect of antioxidants on twitch half relaxation time.
<table>
<thead>
<tr>
<th></th>
<th>CONTROL (10 mM)</th>
<th>TIRON (6.4 mM)</th>
<th>DMSO (500 U/ml)</th>
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<tr>
<td>INITIAL (30 Hz)</td>
<td>17.0 ± 2.3</td>
<td>12.9 ± 1.9**</td>
<td>16.9 ± 1.0</td>
<td>19.4 ± 1.7</td>
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<td>HYPOXIA (30 Hz)</td>
<td>2.6 ± 0.5</td>
<td>3.9 ± 1.1</td>
<td>4.5 ± 0.6</td>
<td>4.9 ± 1.0</td>
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<td>RECOVERY (30 Hz)</td>
<td>6.8 ± 1.7</td>
<td>8.3 ± 0.1</td>
<td>10.6 ± 0.1**</td>
<td>12.5 ± 1.3***</td>
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<td>INITIAL (50 Hz)</td>
<td>23.5 ± 2.6</td>
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<td>23.3 ± 1.5</td>
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<td>8.0 ± 0.8</td>
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<td>16.7 ± 0.0**</td>
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<td>RECOVERY (100 Hz)</td>
<td>17.3 ± 1.5</td>
<td>22.9 ± 1.5**</td>
<td>22.9 ± 1.5**</td>
<td>23.7 ± 1.5**</td>
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</table>

Table 3. Effect of antioxidants on muscle stress during tetanic stimulation.

Effect of antioxidants on muscle stress at 30 Hz, 50 Hz and 100 Hz during initial (normoxia), hypoxia and on reoxygenation (recovery) phases. All data are expressed as N/cm² ± SE; * P = 0.05, ** P < 0.05, *** P < 0.01, compared to control (untreated) tissue.
Figure 10. Effect of antioxidants on max. force (100 Hz) during hypoxia
Figure 11. Effect of antioxidants on recovery of max. force (100 Hz).
Furthermore, antioxidant-treated tissues also recovered significantly, at nearly all frequencies of stimulation, Fig. 11.

DISCUSSION

To our knowledge this is the first study demonstrating the effect of antioxidants, on muscle function in hypoxic skeletal muscle. Our results show that there is significant impairment of skeletal muscle function during hypoxia, characterized by a reduction in force at all frequencies, as well as a decrease of both twitch contraction and relaxation time. The changes in tetanic force can be ameliorated by coincubation with antioxidants. Furthermore, antioxidants improve recovery of hypoxic muscles upon reoxygenation. These findings suggest that oxidants, oxidizing agents or free radicals are produced in skeletal muscle during hypoxia and contribute to decreased force production.

Potential sources of reactive oxygen species during hypoxia

There are a number of potential sources for reactive oxygen species in hypoxia. One source may be mitochondria (9,21,22,26). It has been suggested that even under normal aerobic conditions, about 2% of the total mitochondrial oxygen consumption is shuttled toward the formation of O$_2^\cdot$ This is believed to be generated at complex I and complex III of the electron transport chain (9). During hypoxia, less O$_2$ is available to be reduced to H$_2$O at cytochrome oxidase resulting in an accumulation of reducing equivalents within the mitochondrial respiratory sequence, a condition known as reductive stress (21). This can result in the formation of ROS by the auto-oxidation of one or more mitochondrial complexes.
such as the ubiquinone-ubiquinol redox couple (9,21). Khan et. al. (22), in their studies on isolated hepatocytes subjected to similar conditions of hypoxia, showed evidence of reductive stress by demonstrating an increase in cellular NADH/NAD+ ratio. They concluded that hypoxia induced hepatocyte injury in their model from sustained reductive stress and oxygen activation.

It has been shown that even at a tissue Po2 of 1 mm Hg (normal, 35 mm Hg) there is a significant production of free radicals despite the low Michaelis constant (Km) of cytochrome oxidase complex in the mitochondria (30). Our tissues were subjected to hypoxic levels comparable to other studies in hypoxia (22,26,28). In our model, there was an exponential washout of oxygen over time. It took approximately 20 minutes for oxygen to fall to <6 mm Hg (Fig. 2.1). It is conceivable that this initial period of relative hypoxia could result in the formation of oxygen free radicals by direct single electron reduction of the available molecular oxygen to form O2. These ROS upon reaction with cell membranes and polyunsaturated fatty acids could propagate into a self-perpetuating chain-reaction, resulting in a significant accumulation of free radicals (6).

Other possible sources of ROS include activation of certain enzymatic pathways during hypoxia. Xanthine dehydrogenase is known to be present in reasonably high concentration in rat diaphragm (1). Its exact location within skeletal muscles is not clear but it seems to be predominantly located within endothelial cells in the vasculature (1); the latter is abundant in diaphragm. During hypoxia an insufficient supply of ATP can result in the malfunction of ATP-dependent calcium pumps of the cell. The excess calcium ions stimulate the transformation of xanthine dehydrogenase to xanthine oxidase by the action of calcium-activated proteases. Xanthine oxidase catalyzes the breakdown of hypoxanthine (formed as
a catabolic product of adenine nucleotides in increased amounts during hypoxia) into xanthine and uric acid, releasing $\text{O}_2^-$ (15). Reactive oxygen species may also be formed by cytosolic enzymes such as cyclooxygenase. Under hypoxic conditions there is an increased calcium-dependent activation of the enzyme phospholipase A$_2$ which releases arachidonic acid from phospholipids. Cyclooxygenase reacts with this accumulated fatty acid, simultaneously generating singlet oxygen and hydroxyl radical (OH) (7).

Finally, hypoxia can also cause increased nitric oxide (NO) production. Recent studies on endothelial cells showed that NO production may be stimulated by elevated levels of cytosolic calcium (20) or by increased activity of constitutive nitric oxide synthase (39) occurring during hypoxia. NO is known to be toxic to the cell by its direct interaction with iron-sulfur proteins, inhibition of ribonucleotide reductase and other enzyme systems, nitrosylation of thiols as well as inhibition of protein synthesis (4). It can also exert indirect toxic effects via peroxynitrite formed by its combination with $\text{O}_2^-$. Peroxynitrite is a potent oxidant with "hydroxyl radical-like activity" and has been shown to oxidize thiols, sulfides, lipids and deoxyribose (4). In this study, antioxidants may have functioned in part by preventing peroxynitrite formation during hypoxia, that is by scavenging superoxide or perhaps by direct antioxidant activity on peroxynitrite itself.

**Muscle function during hypoxia**

During hypoxia, there was a decline in peak twitch tension and tetanic tension, and an acceleration of both twitch contraction time and half relaxation time. The muscle function did not recover completely upon subsequent 30 minute reoxygenation. The decline in muscle
tension during hypoxia and incomplete recovery may have been due to several reasons: 

1) Reactive oxygen species mediated injury to some of the muscle fibers. This may also explain the incomplete recovery of muscle function upon subsequent reoxygenation. 

2) It has been shown in cardiac muscle that during hypoxia there may be an increase in intracellular calcium levels due to inhibition of membrane ATPase and the Na⁺/Ca²⁺ exchanger (13). The excess calcium ions can activate proteases which destroy the sarcolemma and cytoskeleton as well as cause breakdown of membrane phospholipids by the action of calcium-activated phospholipases (19). Therefore, function may be reduced by calcium-induced cell injury. 

3) Several changes in the intracellular milieu occur under conditions of hypoxia or fatigue including a rise in inorganic phosphate, decline in pH, a fall in phosphocreatine levels and changes in the concentrations of adenine nucleotides. These changes can directly depress the contractile apparatus, causing a decline in muscle function (14).

The cause of acceleration of twitch kinetics during hypoxia is unclear. Green et. al (17) showed an increased activity of sarcoplasmic reticulum ATPase (SR ATPase) in both slow and fast twitch skeletal muscle during ischemia. This could result in increased reuptake of calcium by the SR ATPase, resulting in faster relaxation. Alternatively, during hypoxia there may be a reduction in the contribution from slow twitch oxidative fibers, which depend more on oxygen for energy metabolism. In contrast, fast twitch glycolytic fibers would be expected to be more preserved in hypoxic muscle as glycolytic pathways could continue to provide energy. This could result in a predominant fast twitch fiber influence on force. Interestingly, slow twitch oxidative fibers are rich in mitochondria which may, as mentioned, represent a source of ROS in hypoxia.
Effect of specific antioxidants on muscle function

Our results indicate that both small (intracellular) and large (extracellular) antioxidants have equal effects on ameliorating the decline in muscle tension during hypoxia and enhancing recovery of tissue function upon reoxygenation. The mechanism of action may or may not have been the same for each of these antioxidants.

N-acetylcysteine, a well known antioxidant in ischemia-reperfusion models, is a low molecular weight antioxidant having a nucleophilic sulfhydryl group which can interact with and detoxify reactive electrophiles and free radicals directly via conjugation or reduction reactions. It has been shown to scavenge OH and hypochlorous acid; its antioxidant effect on other ROS is unlikely. Its indirect effects involve raising intracellular concentration of cysteine, thereby supporting glutathione (an endogenous antioxidant) biosynthesis (2,35). In addition, NAC has been shown to increase the activity of other endogenous intracellular antioxidant enzymes such as superoxide dismutase (25). Recent in vivo and in vitro studies on skeletal muscle (10,23,36) have shown that NAC inhibits skeletal muscle fatigue in both human and animal models. The exact mechanism by which NAC influences the contractile process in muscle is unknown. Khawli et al. (23) proposed that improved contractility and inhibition of fatigue seen with NAC treatment could be due to its effect on SR. During oxidative stress the sulfhydryl groups on the calcium release channels and SR ATPase can be oxidized, thereby causing loss of calcium homeostasis in the contractile apparatus. This may be prevented or reversed by treatment with a free radical scavenger and thiol donor such as NAC (23).
Previous studies (31,33,37) on the diaphragm have shown both DMSO and SOD to be effective antioxidants under conditions of diaphragmatic fatigue. DMSO is a commonly used, highly permeable nonenzymatic sulfur-containing antioxidant and is primarily a \( \text{OH} \) scavenger, although it can also scavenge other ROS (3). Its mechanism of action on the contractile process is believed to be similar to NAC by its action on the SR (32). SOD, on the other hand, primarily catalyzes the dismutation of \( \text{O}_2^- \) to hydrogen peroxide and oxygen. It is a high molecular weight metalloenzyme and so, may be restricted to an extracellular distribution (31). Radak et al. (29) demonstrated that administration of SM-SOD (a poly-(styrene-co-maleic acid) butyl ester linked derivative of SOD) to rats resulted in reduced oxidative damage to skeletal muscles during exhaustive exercise. They showed a significant reduction in plasma and skeletal muscle thiobarbituric acid-reactive substances and reduced plasma xanthine oxidase activity after exercise in the treated group. In our model, it is likely that SOD prevented decline in muscle function during hypoxia by a combination of the above effects.

Tiron® is a less well known antioxidant and to our knowledge its effects have so far not been studied on skeletal muscle. It is a low molecular weight phenolic compound and has been shown to scavenge \( \text{OH}, \text{O}_2^- \) and hydrogen peroxide (18,24). It has been used for in vivo animal studies as a non-toxic metal chelator (24). In addition, it can be used as a spin trap. Upon combining with free radicals, it is easily oxidized to its semiquinone radical, which can be detected by electron paramagnetic resonance imaging (24). We have also shown that
Tiron® inhibits diaphragmatic fatigue similar to other antioxidants (unpublished data). In this study, the improvement in muscle function on coincubation with Tiron® is perhaps due to its free radical scavenging and metal chelating properties.

Conclusions

Hypoxia results in skeletal muscle dysfunction which can be partially prevented by treatment with antioxidants. We speculate that hypoxia results in the production of reactive oxygen or other oxidizing species through a mechanism initiated by reductive stress. These findings suggest a new role of antioxidants in providing protection during conditions of reductive stress, much as they have been shown to provide protection in oxidative stress. This may apply to the proven effectiveness of antioxidant treatment in many other tissue beds and clinical conditions in which problems with oxygen delivery and utilization are present. For example, cell dysfunction caused by ischemia, sepsis or intense metabolic activity seen in fatiguing skeletal muscle have all been shown to be partially prevented by treatment with antioxidants and are likely to represent conditions of a high reducing environment within the cells. Therefore, the traditional view that antioxidants protect tissue from reactive oxygen produced during reperfusion or reoxygenation must be expanded to include the concept of protection during the preceding period of hypoxia or ischemia.
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CHAPTER 3

TIRON (1,2-DIHYDROXYBENZENE-3,5-DISULFONATE) INHIBITS DIAPHRAGM MUSCLE FATIGUE

ABSTRACT

Tiron (1,2-dihydroxybenzene-3,5-disulfonate), a low molecular weight (mol. wt. 332) phenolic compound, has been used in in vivo animal studies as a non-toxic metal chelator. It is also an efficient intracellular antioxidant and has been shown to scavenge superoxide (O₂⁻) radical. In addition, upon combining with different reactive radicals, it is easily oxidized to the EPR-detectable semiquinone radical. The effects of Tiron have not been previously studied on diaphragmatic tissue. Isolated rat diaphragmatic muscle strips were placed in tissue baths containing physiological salt solution. The muscle strips were directly stimulated in an electrical field. After an initial study of the baseline contractile function, the experimental tissues were incubated for 30 minutes in Tiron (10 mM) at 37°C and the effect of Tiron on the contractile properties (twitch characteristics and force-frequency relationships) of the muscle strips was studied. The strips were then rested for 10 minutes following which, they were fatigued over a 4-minute period (20 Hz, 0.33-sec trains, 1 train/sec). Endurance time (time for a 50% decline in contractile function) was measured, as an indicator of muscle

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fatigue. The results indicate that Tiron suppresses low-frequency tetanic tensions similar to other antioxidants. During the fatigue protocol, the endurance time in tissues treated with Tiron was approximately 42% higher than control tissues. Thus, Tiron pretreatment attenuated the rate of development of diaphragmatic fatigue during repetitive isometric tetanic stimulations. In conclusion, the effects of Tiron on diaphragmatic tissue are consistent with that of other previously studied antioxidants.

INTRODUCTION

When skeletal muscles undergo repetitive contractions over a period of time, they undergo a decline in performance, usually seen in the form of decreased twitch tension, decreased maximum tetanic tension and reduced endurance. This phenomenon is called muscle fatigue and is defined as the reversible time-dependent impairment in the capacity to generate force, in response to sustained stimulation (5). The same may also be true with the diaphragm in response to severe work loads, as seen in respiratory failure (2). The exact mechanism of muscle fatigue is still unknown. It has been suggested that free radicals may play an important role in the development of muscle fatigue. Recently, several studies on the diaphragm have given evidence for the involvement of free radicals in fatigue and have demonstrated improvement in muscle function on treatment with antioxidants, such as N-acetylcysteine (NAC), dimethylsulfoxide (DMSO) and superoxide dismutase (SOD) (10,27,34). In a previous study in our laboratory, we evaluated the effects of the antioxidants NAC, DMSO, SOD and Tiron (1,2-dihydroxybenzene-3,5-disulfonate) on muscle function during hypoxia (24). Our results showed significant improvement of function in each of the
antioxidant-treated muscles during hypoxia. Tiron is a superoxide radical scavenger and a metal chelator, whose effects have not been previously studied on muscle fatigue. Since hypoxia and muscle fatigue may share similar cellular changes (15), this study was performed to evaluate if Tiron had effects similar to other antioxidants on muscle fatigue. This study evaluated the effects of Tiron (1,2-dihydroxybenzene-3,5-disulfonate), a superoxide radical scavenger and a metal chelator, whose effects have not been previously studied on muscle fatigue. The results indicate that Tiron has effects on diaphragm muscle function similar to other antioxidants and also inhibits diaphragm muscle fatigue.

METHODS

Surgical procedure and muscle strip preparation

The experiments were conducted on diaphragm muscle strips taken from adult male Sprague Dawley rats (300-500 g). The animals (n = 5) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg /kg), tracheotomized and mechanically ventilated. The phrenic nerve was severed and two diaphragm strips (approximately 0.5 cm) were dissected out from each animal, with portions of the last two ribs and central tendon intact. The muscle strips were placed in separate tissue baths containing physiological salt solution (in meq/l: 21 NaHCO₃, 0.9 NaSO₄, 1.2 NaHPO₄, 0.9 MgCl₂, 2.25 CaCl₂, 5.9 KCl, and 109 NaCl and 2.07 g/l glucose). The baths were maintained at 37°C and were continuously bubbled with 95%O₂-5%CO₂.

The strips were mounted vertically with the central tendon positioned superiorly and attached to a force transducer which was connected to a micropositioner. D-tubocurarine (10
μM) was added to eliminate activation of intramuscular nerve branches. The diaphragm strips were positioned between two platinum plates and stimulated by direct electrical field.

**Stimulation paradigm**

After the strips were mounted in tissue chambers, the maximum stimulation current was determined by gradually increasing the current until a maximum was reached corresponding to peak twitch tension. At this maximum current, (L<sub>0</sub>) was then determined as the optimal length (by adjusting the micropositioner between intermittent stimulations), at which maximum twitch tension was obtained. Thereafter, all stimulations were performed at L<sub>0</sub> and at maximal current.

**Study of muscle function**

The muscle strips were tetanically stimulated at 20 to 150 Hz, with an interval of ~30 seconds between each tetanic stimulation; pulses were of 0.2 ms duration, with a train duration of 400 ms. After an initial study of muscle function, the experimental tissue was treated with 10 mM Tiron for half-an-hour, following which muscle function was assessed by repeating a twitch contraction and force-frequency stimulation. After 10 minutes rest, the muscle was fatigued with 20 Hz stimulus for 4 minutes at 1 contraction per second and a duration of 330 msec. The endurance time of the treated and control muscles were compared. Endurance time was defined as time taken for the reduction of muscle tension (following fatiguing stimulus) to half its initial tension. Finally, recovery of muscle function was assessed at 15 minutes post-fatigue.
Data Analysis

At the end of the experiment, the length and wet weight of each muscle strip was obtained. For conversion of force to absolute stress, the cross-sectional area of each diaphragm strip was determined by dividing the muscle mass (weight in gm.) by the product of muscle length in centimeters and muscle density (assumed to be 1.06 g/cm$^3$) (8). All values are expressed as group means ± SEM. Data were analyzed by SAS JMP (SAS Institute Inc., Cary, NC) using repeated measures ANOVA, with the experimental animal being treated as a random variable. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of Tiron on baseline muscle function

We compared the force-frequency relationship of 20 Hz - 100 Hz stimuli, of control and Tiron-treated muscles. Tiron tended to suppress tetanic tensions at low-frequencies (20 - 40 Hz). However, this was not statistically significant (Fig. 12). The suppression of low-frequency tetanic tensions has also been observed with other antioxidants (20,29,33).

Effect of Tiron on muscle fatigue

As seen in Fig. 13, Tiron significantly inhibited muscle fatigue ($P < 0.01$). The time taken for muscle tension to reach half its initial tension, or the endurance time, was significantly higher in Tiron-treated muscles ($P < 0.01$), Fig. 14.
Figure 12.

Effect of Tiron on muscle function at different frequencies of stimulation. All data are expressed as % Maximum force (with 100 % = force at 100 Hz stimulation, before treatment). Tiron suppressed muscle function at lower frequencies of stimulation, but was not statistically significant.
Figure 12.
Figure 13.

Effect of Tiron on muscle fatigue. The muscle was fatigued at 20 Hz, 330 ms duration, 1 train/sec. Data are represented as % initial force, where initial 100 % = force at the start of fatiguing stimulus. Muscles treated with Tiron (n=5) significantly inhibited muscle fatigue, $P < 0.01$. 
Figure 13.

$P < 0.01$
Effect of Tiron on Endurance time. Endurance time was defined as time taken for the reduction of muscle tension (following fatiguing stimulus) to half its initial tension. Tiron significantly improved endurance time, $P < 0.01$. 

Figure 14.
Figure 14.
Effect of Tiron on postfatigue recovery

Tiron also improved recovery of tetanic tensions at higher frequencies of stimulation (50 - 100 Hz), observed 15 minutes following fatigue, (table 3.1). The recovery of maximum tension (100 Hz) in Tiron-treated muscles was significantly higher than control tissues ($P < 0.01$), Fig. 15.

DISCUSSION

To the best of our knowledge, this is the first study to evaluate the role of Tiron on diaphragm muscle fatigue. We also studied the effect of Tiron on baseline muscle function as well as on recovery of muscle function following fatigue. Our results show that Tiron inhibits diaphragm muscle fatigue. Furthermore, Tiron improved recovery of muscle function following fatigue.

Free radicals and muscle fatigue

Recently, several studies on the diaphragm and other skeletal muscles have given evidence for the involvement of free radical species in muscle fatigue (1,2,6,7,9,11,19). One of the earliest pieces of evidence for free radical involvement during muscular exhaustion came from the studies of Davies et al. (9) who showed increased free radical production using spin resonance technique. Several studies on the diaphragm (2,6,7,11,33) have shown that free radicals are clearly involved in the diaphragmatic dysfunction seen during its repeated stimulation.
Figure 15.

Effect of Tiron on post-fatigue recovery. Recovery of muscle function was assessed at 15 minutes following fatigue. Data are represented for 100 Hz stimulation, as a % of tension recorded before treatment with drug (initial tension). Tiron significantly improved recovery of muscle function, $P < 0.05$. 
Figure 15.
Table 4.

Effect of Tiron on post-fatigue recovery. Data represents recovery of muscle function 15 minutes following fatigue. All data are expressed in % Maximum ± SEM (with initial = 100 %, before fatiguing stimuli). Tiron-treated muscle recovered significantly at maximum frequency of simulation (100 Hz), *$P < 0.05$. 
Post-fatigue recovery:

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<th>FREQUENCY</th>
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<td>15.96 ± 3.8</td>
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<td>38.08 ± 3.2</td>
<td>41.73 ± 5.6</td>
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<tr>
<td>100 Hz</td>
<td>56.33 ± 3.8</td>
<td>72.79 ± 5.9 *</td>
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Table 4.
It is known that even normally functioning muscles at rest are capable of producing free radicals. However, the production of free radicals increases several fold on prolonged or intense muscular contraction (27). Likewise, there is increasing evidence that free radicals may be produced during repetitive contractions of the diaphragm (11). Anzueto et al. demonstrated an increased production of thiobarbituric acid-reactive substances (TBARS) and glutathione content in rat diaphragm and provided evidence of lipid peroxidation products due to free radical damage with simultaneous activation of the glutathione redox cycle (1). Previous studies in our laboratory by Diaz et. al. have demonstrated the generation of hydroxyl radical during diaphragmatic stimulation by using salicylate as the specific hydroxyl radical scavenger (11). N-acetylcysteine (NAC), which is an anti-oxidant and is structurally similar to glutathione, has been shown to attenuate diaphragmatic dysfunction (10,33).

Previous in vitro and in vivo studies have shown that antioxidants such as NAC, DMSO and SOD inhibit diaphragm muscle fatigue (10,27,30,34). This study was performed to determine if Tiron had effects similar to other antioxidants on muscle fatigue. Tiron is a less well known low molecular weight phenolic antioxidant. It has been shown to scavenge OH, O$_2^{*-}$ and hydrogen peroxide (H$_2$O$_2$) (25). Upon combining with O$_2^{*-}$ it is oxidized to a semiquinone, which is a stable free radical compound. The semiquinone radical can be easily detected using electron paramagnetic resonance (EPR) imaging. Therefore, Tiron has also been used as a spin trap to detect free radicals (21). Furthermore, Tiron has been shown to inhibit lipid peroxidation, as well as protect the activity of other endogenous antioxidants such as SOD and catalase (23,36). It also protects against DNA damage induced by free radicals.
In addition, Tiron has been used for in vivo animal studies as a non-toxic metal chelator (13,25). It has been used as an antidote against heavy metal toxicity (4). Administration of Tiron has been shown to protect against teratogenic toxicity induced by certain heavy metals such as vanadium and uranium by mobilizing tissue bound metal and inducing its excretion (12,13).

In this study, the improvement in muscle function on coincubation with Tiron is perhaps due to its free radical scavenging and metal chelating properties. Because of its low molecular weight, Tiron easily localizes at critical intracellular sites and can thus protect against harmful effects of locally generated $\cdot O_2$ and $H_2O_2$ (25). This offers advantage over the more commonly used $O_2^\cdot$ scavenger, SOD, which is largely restricted to the extracellular space because of its large molecular weight.

Sources of reactive species during muscle fatigue

Several potential sites of reactive species generation have been suggested during diaphragm fatigue. The excessive metabolic activity during muscle fatigue can result in accelerated mitochondrial respiration, simultaneously releasing large amounts of $O_2^\cdot$ during the process (3,27). $O_2^\cdot$ upon dismutation can result in the formation of another oxidant, $H_2O_2$. Furthermore, in the presence of transition metals such as iron ($Fe^{2+}$), $O_2^\cdot$ and $H_2O_2$ can combine to form hydroxyl radical (Fenton reaction), which is a very potent oxidant (22). Reactive species, during intense exercise, have also been shown to be produced through enzymatic pathways such as, cyclooxygenase and xanthine oxidase (16,18,26,31,35). Other sources may include oxidases in the sarcoplasmic reticulum (23) and sarcolemma (32).
Mechanism of muscle fatigue by reactive species

The exact site of action of reactive species in causing muscle fatigue is unknown. However, several targets including cell membrane and the contractile apparatus have been proposed. Oxidation of membrane lipids can result in a process called lipid peroxidation characterized by formation of lipid peroxides and peroxyl radicals (14). The latter can propagate into additional chain reactions, resulting in the generation of a large amount of free radicals (14). Khawli et al. proposed oxidation of sarcoplasmic reticulum (SR) by reactive oxygen species, which can result in increased intracellular calcium and decreased SR calcium content during fatigue (20). They showed inhibition of muscle fatigue in muscles treated with NAC, which can reverse sulfhydryl oxidation of SR. Finally, reactive species may also alter cellular redox state thereby, compromising function of the contractile apparatus (28).

Inhibition of muscle fatigue by antioxidants

The exact mechanism of action of antioxidants is unknown; however, several mechanisms have been proposed. In addition to scavenging and thereby preventing injury by specific free radicals, antioxidants may play an important role by preserving redox balance of the cell (28). Furthermore, antioxidants such as NAC can reverse sulfhydryl oxidation of critical proteins as well as replenish stores of endogenous antioxidant glutathione (10,20,33). In a previous study, we showed that the antioxidants, NAC, DMSO, SOD and Tiron protected muscle function during hypoxia (24). Interestingly, several common changes in the intracellular milieu occur under conditions of hypoxia or fatigue of a muscle. These include,
a rise in inorganic phosphate, decline in pH, a fall in phosphocreatine levels and changes in the concentrations of adenine nucleotides, all of which can directly depress the contractile apparatus, causing a decline in muscle function (15). It is possible that antioxidants protect muscle function during fatigue and hypoxia by preventing these metabolic alterations by directly influencing metabolic pathways. Tiron may have protected muscle function by any of the above proposed antioxidant mechanisms and/or by preventing Fenton chemistry by chelating metals.

Likewise, similar to other antioxidants (20,29,33), Tiron also suppressed baseline muscle function at low frequencies as well as improved recovery of muscle function following fatigue. However, the muscles did not recover completely following fatigue which may have been due to some long term damage to muscle fibers during fatigue. Furthermore, there was a greater recovery of high-frequency stimulation following fatigue, compared to low-frequency stimulation. These phenomena have also been observed by other studies on diaphragm fatigue (1,10,17,32,34), and have been attributed to free radical mediated damage to membrane lipids, contractile proteins, enzymes or other cellular organelles.

Conclusions

Tiron inhibits diaphragm muscle fatigue and improves endurance time in the fatigued diaphragm. Furthermore, it significantly improves recovery of maximum muscle tension following fatigue. The mechanism of action may be a combination of its superoxide scavenging and metal chelating properties.
LIST OF REFERENCES


CHAPTER 4

NITRIC OXIDE MODULATES SKELETAL MUSCLE FUNCTION DURING HYPOXIA

ABSTRACT

Reactive oxygen species and nitric oxide are known to modulate skeletal muscle contractile activity under physiological conditions. However, the role of nitric oxide on skeletal muscle function during hypoxia is not well known. In this study, we evaluated the dose-dependent effects of a nitric oxide synthase inhibitor (L-NAME), a long acting nitric oxide donor (DETA/NO) and the guanylate cyclase inhibitor (ODQ), on rat diaphragm muscle function during hypoxia. The muscle strips were placed in tissue baths containing physiological salt solution at 37°C and were directly stimulated in an electrical field. Muscle function was recorded before treatment (initial), after 30 minutes of incubation with the drug (baseline), during hypoxia and on reoxygenation (recovery). The tissue baths were bubbled with 95%O₂, 5%CO₂ during initial baseline and recovery, and with 95%N₂, 5%CO₂ during hypoxia. Our results indicate that both L-NAME (1 μM - 10 mM) and DETA/NO (1 μM -
100 µM) significantly attenuated the decline in muscle function during hypoxia \((P < 0.05)\).

Also, L-NAME significantly improved recovery of muscle function \((P < 0.05)\), upon reoxygenation. However, ODQ did not have any effect on muscle function compared to control tissue. In conclusion, both endogenous nitric oxide (produced by skeletal muscle) as well as exogenous nitric oxide protect skeletal muscle function during hypoxia. Furthermore, these effects are likely to be produced via a non-cGMP mediated pathway.

**INTRODUCTION**

Endothelium Derived Relaxing Factor, later identified in 1987 as nitric oxide (NO) has been classically identified as a smooth muscle relaxant \((11,33)\). It has since been known to mediate several important biological phenomena such as, inhibition of platelet aggregation, macrophage-mediated cell killing, regulation of basal blood pressure, penile corpus cavernosum relaxation, long-term memory development, regulation of glomerular microcirculation, ventilation-perfusion matching and prevention of pylorospasm in infantile pyloric stenosis \((22,26,32,43,52)\).

Recently, increasing evidence has suggested an important role of nitric oxide in the modulation of skeletal muscle function, under physiological conditions \((3,12,24,28,39,44,49)\). However, the influence of \(\cdot\) NO on skeletal muscle contractile function under hypoxic conditions is not well known. The study of diaphragm muscle function during hypoxia may be clinically relevant particularly under conditions of respiratory failure and associated cardiovascular collapse and resuscitation, where conditions are appropriate for local hypoxia.
and ischemia-reperfusion to occur. In a previous chapter, a protective role of antioxidants on muscle function during hypoxia as well as on reoxygenation was demonstrated, suggesting a role of free radicals under such conditions (25). In addition, other studies have shown increased production of \('NO\) in different tissues during hypoxia (15,21,59). The influence of \('NO\) on diaphragm function has been demonstrated in models of septic shock where tissue hypoxia is prevalent in a setting of increased \('NO\) production from \(\text{iNOS}\) (inducible nitric oxide synthase) (14). Furthermore, in a study on heart muscle, Depre et. al. demonstrated that inhibition of nitric oxide synthase preserves energy metabolism in ischemia (8). This study was performed to evaluate if similar mechanisms existed in hypoxic skeletal muscle.

The experiments evaluated the role of nitric oxide in modulating muscle function during normoxia, hypoxia and upon reoxygenation. We studied the effect of basally released \('NO\) on skeletal muscle contractility by blocking its production using a nitric oxide synthase inhibitor, \(\text{N}^\text{G}-\text{Nitro-L-arginine-methyl ester hydrochloride (L-NAME)}\). Second, we evaluated the effect of acute increases in exogenous nitric oxide on skeletal muscle function, using a long acting \('NO\) donor, \((Z)-1-[2\text{-Aminoethyl})-\text{N-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate (DETA/NO)}\). And lastly, to determine if the effects of basal \('NO\) on muscle function are mediated via the cGMP pathway, the effects of inhibiting this pathway were studied using a specific guanylate cyclase inhibitor, \(1H-[1,2,4]\text{oxadiazolo[4,3-}\alpha\text{]quinoxalin-1-one (ODQ)}\).

The results indicate a protective role of both L-NAME as well as DETA/NO on muscle function during hypoxia. In addition, L-NAME also improved recovery of muscle function during reoxygenation. However, ODQ did not have any effect on muscle function.
METHODS

*Surgical procedure and muscle strip preparation*

The experiments were conducted on diaphragm muscle strips taken from adult male Sprague Dawley rats (300-500 g). The animals (n ≥ 9, for each drug) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg /kg), tracheotomized and mechanically ventilated. The phrenic nerve was severed and four diaphragm strips (approximately 0.5 cm) were dissected out with portions of the last two ribs and central tendon intact. The muscle strips were placed in separate tissue baths containing physiological salt solution (in meq/l: 21 NaHCO₃, 0.9 NaSO₄, 1.2 NaHPO₄, 0.9 MgCl₂, 2.25 CaCl₂, 5.9 KCl, and 109 NaCl and 2.07 g/l glucose). The baths were maintained at 37°C and were continuously bubbled with 95%O₂-5%CO₂ during initial and prehypoxic phases and 95%N₂-5%CO₂ during the hypoxic phase. We have previously shown that, during the hypoxic phase, the PO₂ in the baths drops rapidly, reaching <50 mmHg within 3 minutes, from the initial phase (our unpublished data).

The strips were mounted vertically with the central tendon positioned superiorly and attached to a force transducer which was connected to a micropositioner. D-tubocurarine (10 μM) was added to eliminate activation of intramuscular nerve branches. The diaphragm strips were positioned between two platinum plates and stimulated by direct electrical field.
Stimulation paradigm

After the strips were mounted in the tissue chamber, the maximum required stimulation current and the force-optimal length ($L_o$) were determined by adjusting the micropositioner between intermittent stimulations. All stimulations were performed at $L_o$ and at supra-maximal current.

Study of muscle function

The muscle strips were tetanically stimulated at 20 to 150 Hz, with an interval of ~30 seconds between each tetanic stimulation; pulses were of 0.2 ms duration, with a train duration of 400 ms.

Experimental protocol

After $L_o$ measurement, the tissues were rested for about 10 minutes. Then initial twitch and force-frequency measurements (without drug treatment) were taken. One muscle strip served as a control tissue and the remaining three were experimental tissues. The control tissue was bathed in physiological saline throughout the length of the experiment, while the experimental tissues were each bathed in a different dose of the drug under study.

We studied the dose-dependent effects of the NOS inhibitor, L-NAME (1 $\mu$M, 10 $\mu$M, 100 $\mu$M, 1 mM and 10 mM), an NO donor, DETA/NO (1 $\mu$M, 10 $\mu$M and 100 $\mu$M) and a guanylate cyclase inhibitor, ODQ (10 nM, 100 nM and 1000 nM). A pre-hypoxic run was taken after 30 minutes incubation in the drug, in order to study the
effect drug on muscle function. The baths were then subjected to 30 minutes hypoxia and finally, 30 minutes reoxygenation, with study of muscle function at the end of each phase.

**Data Analysis**

At the end of the experiment, the length and wet weight of each muscle strip was obtained. For conversion of force to absolute stress, the cross-sectional area of each diaphragm strip was determined by dividing the muscle mass (weight in gm.) by the product of muscle length in centimeters and muscle density (assumed to be 1.06 g/cm$^3$). All values are expressed as group means ± SE. Data were analyzed by SAS JMP (SAS Institute Inc., Cary, NC) using repeated measures ANOVA. For twitch kinetic measurements, the dependent variable was ‘time’ and the independent variables were ‘treatment’ and ‘animal’. For twitch and tetanic tension measurements, the dependent variable was ‘force’ and the independent variables were ‘treatment’ and ‘animal’. All tetanic force measurements were analyzed as a fraction of their initial (normoxic) force values. A $P < 0.05$ was considered to be statistically significant.
RESULTS

Effect of L-NAME on muscle function

a) Twitch characteristics:

i) Peak twitch tension

There was a significant fall in peak twitch tension during normoxia, at the end of half-hour incubation in control tissue and L-NAME (1 mM)-treated tissue, $P < 0.05$, Table 5. During hypoxia there was a significant fall in peak twitch tension in all tissues, compared to the initial normoxic phase ($P < 0.001$). Furthermore, in all tissues peak twitch tension did not recover to initial tension upon reoxygenation, $P < 0.001$. There was no difference in peak twitch tension between the tissues (control and different doses) within each gas phase.

ii) Time to peak tension

Time to peak tension remained unchanged during normoxic incubation in control and L-NAME-treated tissues, Table 6. However, during hypoxia, there was a significant reduction in time to peak tension, as compared to the initial normoxic phase, and it did not recover significantly upon reoxygenation ($P < 0.001$). There was no difference in time to peak tension between the tissues (control and different doses) within each gas phase.
**L-NAME**

**PEAK TWITCH TENSION:**

<table>
<thead>
<tr>
<th>DRUG</th>
<th>INITIAL (N/cm²) ± SEM</th>
<th>PRE-HYPOXIA (N/cm²) ± SEM</th>
<th>HYPOXIA (N/cm²) ± SEM</th>
<th>RECOVERY (N/cm²) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.03 ± 0.4</td>
<td>6.74 ± 0.3*</td>
<td>1.74 ± 0.2**</td>
<td>2.26 ± 0.1**</td>
</tr>
<tr>
<td>L-NAME (1 μM)</td>
<td>7.01 ± 0.5</td>
<td>5.95 ± 0.6</td>
<td>2.17 ± 0.3**</td>
<td>1.80 ± 0.3**</td>
</tr>
<tr>
<td>L-NAME (10 μM)</td>
<td>7.68 ± 0.6</td>
<td>6.81 ± 0.5</td>
<td>2.18 ± 0.2*8</td>
<td>2.58 ± 0.2**</td>
</tr>
<tr>
<td>L-NAME (100 μM)</td>
<td>7.51 ± 0.7</td>
<td>6.48 ± 0.7</td>
<td>2.38 ± 0.3**</td>
<td>2.16 ± 0.5**</td>
</tr>
<tr>
<td>L-NAME (1 mM)</td>
<td>8.10 ± 0.4</td>
<td>6.98 ± 0.5*</td>
<td>1.94 ± 0.2**</td>
<td>2.62 ± 0.2**</td>
</tr>
<tr>
<td>L-NAME (10 mM)</td>
<td>7.12 ± 0.5</td>
<td>6.67 ± 0.4</td>
<td>2.13 ± 0.4**</td>
<td>3.10 ± 0.4**</td>
</tr>
</tbody>
</table>

*P < 0.05, compared to initial phase, **P < 0.001, compared to initial phase.

Table 5. Initial phase: baseline muscle function before treatment with drug in normoxia (95%O₂, 5%CO₂). Pre-hypoxic phase: muscle function at the end of half-hour incubation with drug during normoxia. Hypoxic phase: muscle function during hypoxia (95%N₂, 5%CO₂). Recovery phase: muscle function following reoxygenation (95%O₂, 5%CO₂).
**L-NAME**

**TIME TO PEAK TENSION:**

<table>
<thead>
<tr>
<th>DRUG</th>
<th>INITIAL msec ± SEM</th>
<th>PRE-HYPOXIA msec ± SEM</th>
<th>HYPOXIA msec ± SEM</th>
<th>RECOVERY msec ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.1 ± 0.1</td>
<td>30.2 ± 0.1</td>
<td>18.2 ± 0.1*</td>
<td>22.6 ± 0.1*</td>
</tr>
<tr>
<td>L-NAME (1 µM)</td>
<td>32.0 ± 0.1</td>
<td>33.8 ± 0.2</td>
<td>18.0 ± 0.1*</td>
<td>22.1 ± 0.1*</td>
</tr>
<tr>
<td>L-NAME (10 µM)</td>
<td>32.7 ± 0.1</td>
<td>32.0 ± 0.1</td>
<td>18.7 ± 0.1*</td>
<td>23.0 ± 0.1*</td>
</tr>
<tr>
<td>L-NAME (100 µM)</td>
<td>30.5 ± 0.2</td>
<td>31.9 ± 0.2</td>
<td>17.7 ± 0.1*</td>
<td>22.8 ± 0.1*</td>
</tr>
<tr>
<td>L-NAME (1 mM)</td>
<td>31.3 ± 0.1</td>
<td>29.7 ± 0.1</td>
<td>17.4 ± 0.1*</td>
<td>23.3 ± 0.1*</td>
</tr>
<tr>
<td>L-NAME (10 mM)</td>
<td>28.3 ± 0.1</td>
<td>29.3 ± 0.1</td>
<td>17.9 ± 0.1*</td>
<td>23.4 ± 0.1*</td>
</tr>
</tbody>
</table>

*P < 0.001, compared to initial phase.

Table 6. **Initial phase:** baseline muscle function before treatment with drug in normoxia (95%O₂, 5%CO₂). **Pre-hypoxic phase:** muscle function at the end of half-hour incubation with drug during normoxia. **Hypoxic phase:** muscle function during hypoxia (95%N₂, 5%CO₂). **Recovery phase:** muscle function following reoxygenation (95%O₂, 5%CO₂).
iii) Half-relaxation time

There was a significant prolongation in half-relaxation time in control and L-NAME-treated (10 μM, 100 μM, 1 mM) tissues, during ½ hr normoxic incubation, $P < 0.01$, Table 7. However, during the hypoxic phase, twitch half-relaxation time was significantly reduced in all tissues, $P < 0.001$. Furthermore, it did not recover completely upon reoxygenation, $P < 0.001$. Similar to the other twitch parameters, there was no difference in half relaxation time between the tissues (control and different doses) within each gas phase.

b) Force-frequency relationships:

i) Effect of L-NAME on muscle function during normoxia

L-NAME (1 μM - 10 mM) did not have any effect on muscle function (low and high frequency stimulation) during the half-hour incubation in the drug at various concentrations during normoxia, Fig. 16.

ii) Effect of L-NAME on muscle function during hypoxia

During hypoxia, there was a significant fall in muscle tension at all frequencies of stimulation ($P < 0.001$), not shown. L-NAME significantly attenuated the decline in muscle during hypoxia at both low and high frequencies of stimulation, $P < 0.05$, Fig. 17 and Fig. 18. However, not all dose-effects reached statistical significance.

iii) Effect of L-NAME on muscle function during recovery

Upon reoxygenation, L-NAME improved recovery of muscle function only during
Table 7. Initial phase: baseline muscle function before treatment with drug in normoxia (95%O₂, 5%CO₂). Pre-hypoxic phase: muscle function at the end of half-hour incubation with drug during normoxia. Hypoxic phase: muscle function during hypoxia (95%N₂, 5%CO₂). Recovery phase: muscle function following reoxygenation (95%O₂, 5%CO₂).
Figure 16. Effect of L-NAME on muscle function during normoxia, as compared to % maximum initial force, where maximum initial (before treatment) force at 100 Hz = 100 %
Figure 17. Effect of L-NAME during hypoxia, 30 Hz stimulation
**Figure 18. Effect of L-NAME during hypoxia, 100 Hz stimulation**

- Control
- L-NAME, 1 μM
- L-NAME, 10 μM
- L-NAME, 100 μM
- L-NAME, 1000 μM
- L-NAME, 10,000 μM

*P < 0.05*
Figure 19. Effect of L-NAME on recovery, 30 Hz stimulation

*P < 0.001
Figure 20. Effect of L-NAME on recovery, 100 Hz stimulation
low-frequency stimulation, Fig. 19 and Fig. 20. Furthermore, on post hoc analysis, the significant effect of L-NAME was seen only with the highest dose of treatment (10 mM) at 30 Hz stimulation, Fig. 19.

**Effect of DETA/NO on muscle function**

*a) Twitch characteristics:*

*i) Peak twitch tension*

There was a significant reduction in peak twitch tension with time, during the half-hour incubation in normoxia, in the control and DETA/NO-treated (1 µM) tissue, \( P < 0.05 \), Table 8. Peak twitch tension declined significantly during hypoxia and also did not completely recover upon reoxygenation, \( P < 0.001 \). There was no difference in peak twitch tension between the tissues (control and different doses) within each gas-phase.

*ii) Time to peak tension*

The time to peak tension did not change during half-hour incubation of tissue in normoxia (pre-hypoxic phase), except in 100 µM DETA/NO treated tissue, where it decreased significantly, \( P < 0.05 \), Table 9. During hypoxia, time to peak tension declined significantly in all tissues, \( P < 0.002 \), and did not recover completely upon reoxygenation. There was no difference in time to peak tension between the tissues (control and different doses) within each gas phase.
DETA/NO

PEAK TWITCH TENSION:

<table>
<thead>
<tr>
<th>DRUG</th>
<th>INITIAL (N/cm²) ± SEM</th>
<th>PRE-HYPOXIA (N/cm²) ± SEM</th>
<th>HYPOXIA (N/cm²) ± SEM</th>
<th>RECOVERY (N/cm²) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.01 ± 0.6</td>
<td>6.30 ± 0.5*</td>
<td>1.91 ± 0.3**</td>
<td>2.39 ± 0.3**</td>
</tr>
<tr>
<td>DETA/NO (1 μM)</td>
<td>7.14 ± 0.7</td>
<td>5.49 ± 0.6*</td>
<td>1.35 ± 0.2**</td>
<td>2.20 ± 0.3**</td>
</tr>
<tr>
<td>DETA/NO (10 μM)</td>
<td>6.53 ± 1.0</td>
<td>5.49 ± 0.8</td>
<td>2.26 ± 0.5**</td>
<td>2.42 ± 0.3**</td>
</tr>
<tr>
<td>DETA/NO (100 μM)</td>
<td>6.00 ± 0.7</td>
<td>5.23 ± 0.8</td>
<td>2.00 ± 0.2**</td>
<td>1.78 ± 0.4**</td>
</tr>
</tbody>
</table>

*P < 0.05, compared to initial phase. **P < 0.001, compared to initial phase

Table 8. Initial phase: baseline muscle function before treatment with drug in normoxia (95%O₂, 5%CO₂). Pre-hypoxic phase: muscle function at the end of half-hour incubation with drug during normoxia. Hypoxic phase: muscle function during hypoxia (95%N₂, 5%CO₂). Recovery phase: muscle function following reoxygenation (95%O₂, 5%CO₂).
**DETA/NO**

**TIME TO PEAK TENSION:**

<table>
<thead>
<tr>
<th>DRUG</th>
<th>INITIAL msec ± SEM</th>
<th>PRE-HYPOXIA msec ± SEM</th>
<th>HYPOXIA msec ± SEM</th>
<th>RECOVERY msec ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.2 ± 0.2</td>
<td>30.5 ± 0.2</td>
<td>17.7 ± 0.1**</td>
<td>24.7 ± 0.2*</td>
</tr>
<tr>
<td>DETA/NO (1 µM)</td>
<td>29.8 ± 0.1</td>
<td>30.8 ± 0.3</td>
<td>17.8 ± 0.2**</td>
<td>25.7 ± 0.2</td>
</tr>
<tr>
<td>DETA/NO (10 µM)</td>
<td>30.4 ± 0.1</td>
<td>27.8 ± 0.2</td>
<td>19.0 ± 0.1**</td>
<td>24.0 ± 0.1*</td>
</tr>
<tr>
<td>DETA/NO (100 µM)</td>
<td>29.0 ± 0.1</td>
<td>25.5 ± 0.2*</td>
<td>18.3 ± 0.1**</td>
<td>20.3 ± 0.1**</td>
</tr>
</tbody>
</table>

*P < 0.05, compared to initial phase. **P < 0.001, compared to initial phase.

Table 9. *Initial phase:* baseline muscle function before treatment with drug in normoxia (95%O₂, 5%CO₂). *Pre-hypoxic phase:* muscle function at the end of half-hour incubation with drug during normoxia. *Hypoxic phase:* muscle function during hypoxia (95%N₂, 5%CO₂). *Recovery phase:* muscle function following reoxygenation (95%O₂, 5%CO₂).
iii) **Half-relaxation time**

There was a significant increase in half-relaxation time in all tissues during the half-hour incubation in normoxia, Table 10. During hypoxia, half-relaxation time declined significantly in all tissues, compared to initial phase, $P < 0.05$. On reoxygenation, it recovered significantly in all DETA/NO-treated tissues. However, in the control tissue the half-relaxation time remained significantly smaller, compared to the initial phase, $P < 0.05$. There was no difference in half-relaxation time between the tissues (control and different doses) within each gas phase.

**b) Force-frequency relationships:**

i) **Effect of DETA/NO on muscle function during normoxia**

The effects of incubation of tissue in half-hour normoxia were studied in control and DETA/NO-treated tissues. DETA/NO did not have any effect on muscle function during normoxia at both low and high frequencies of stimulation, Fig. 21.

ii) **Effect of DETA/NO on muscle function during hypoxia**

DETA/NO significantly protected muscle function during hypoxia during both low and high frequencies of stimulation, $P < 0.05$, Fig 22 and Fig 23. These effects were seen only with the higher doses of DETA/NO (10 $\mu$M and 100 $\mu$M).
**DETA/NO**

HALF RELAXATION TIME:

<table>
<thead>
<tr>
<th>DRUG</th>
<th>INITIAL msec ± SEM</th>
<th>PRE-HYPOXIA msec ± SEM</th>
<th>HYPOXIA msec ± SEM</th>
<th>RECOVERY msec ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.3 ± 0.1</td>
<td>37.3 ± 0.2*</td>
<td>17.5 ± 0.1**</td>
<td>25.8 ± 0.2*</td>
</tr>
<tr>
<td>DETA/NO (1 µM)</td>
<td>29.5 ± 0.1</td>
<td>37.7 ± 0.3*</td>
<td>18.8 ± 0.1**</td>
<td>26.0 ± 0.2</td>
</tr>
<tr>
<td>DETA/NO (10 µM)</td>
<td>29.0 ± 0.3</td>
<td>46.6 ± 0.5*</td>
<td>17.8 ± 0.2*</td>
<td>30.0 ± 0.2</td>
</tr>
<tr>
<td>DETA/NO (100 µM)</td>
<td>27.3 ± 0.3</td>
<td>38.5 ± 0.3*</td>
<td>18.8 ± 0.1*</td>
<td>23.3 ± 0.2</td>
</tr>
</tbody>
</table>

*P < 0.05, compared to initial phase. **P < 0.001, compared to initial phase

Table 10. Initial phase: baseline muscle function before treatment with drug in normoxia (95%O₂, 5%CO₂). Pre-hypoxic phase: muscle function at the end of half-hour incubation with drug during normoxia. Hypoxic phase: muscle function during hypoxia (95%N₂, 5%CO₂). Recovery phase: muscle function following reoxygenation (95%O₂, 5%CO₂).
Figure 21. Effect of DETA/NO on muscle function during normoxia, as compared to % maximum initial force, where maximum initial force (before treatment) at 100 Hz = 100%.
Figure 22. Effect of DETA/NO on muscle function during hypoxia, 30 Hz stimulation
Figure 23. Effect of DETA/NO on muscle function during hypoxia, 100 Hz stimulation

- Control
- DETA/NO, 1 μM
- DETA/NO, 10 μM
- DETA/NO, 100 μM

*P < 0.05
**P = 0.07
Figure 24. Effect of DETA/NO on recovery of muscle function, 30 Hz stimulation.
Figure 25. Effect of DETA/NO on recovery of muscle function, 100 Hz stimulation
iii) Effect of DETA/NO on recovery of muscle function

Upon reoxygenation, DETA/NO (1 μM - 100 μM) did not have any effect of recovery of muscle function at both low and high frequencies of stimulation, Fig. 24 and Fig. 25.

Effect of ODQ on muscle function

In the doses tested (10 nM, 100 nM and 1000 nM), ODQ did not have any effect on muscle function during normoxia (Fig. 26), hypoxia (Figs. 27, 28) or on reoxygenation (Figs. 29, 30) at both low and high frequencies of stimulation.

DISCUSSION

In the present study, we evaluated the role of nitric oxide in modulating skeletal muscle function during hypoxia. The advantage of our model is that the effect of NO on muscle function could be studied on isolated rat diaphragm without its confounding effects on an intact circulatory system. Paradoxically, both L-NAME and DETA/NO protected muscle function during hypoxia as well as improved recovery of muscle function upon reoxygenation. There was no effect of ODQ on muscle function suggesting that the effects of endogenous NO are likely to be mediated via cGMP-independent pathways.

The study of the role of NO on skeletal muscle function during hypoxia is important since NO has been shown to influence the function of several important cell
Figure 26. Effect of ODQ on muscle function during normoxia, as compared to % maximum initial force, where maximum initial force (before treatment) at 100 Hz = 100 %.
Figure 27. Effect of ODQ on muscle function during hypoxia, 30 Hz stimulation
Figure 28. Effect of ODQ on muscle function during hypoxia, 100 Hz stimulation
Figure 29. Effect of ODQ on recovery of muscle function, 30 Hz stimulation.
Figure 30. Effect of ODQ on recovery of muscle function, 100 Hz stimulation
organelles and enzyme-systems, including its action on the contractile apparatus, energy metabolism as well as redox-sensitive regulatory proteins (39). Furthermore, there is evidence to suggest increased production of NO during both acute and chronic hypoxia (15,59-61). Studies on endothelial cells subjected to an acute hypoxic environment suggest that increased production of NO was stimulated by elevated levels of cytosolic calcium (15) or, by increased activity of cNOS occurring during hypoxia (59). In addition, several studies have demonstrated that hypoxia alone, without subsequent reoxygenation or reperfusion, can result in the production of oxidants in different tissues (25,34,35,50). This simultaneous presence of reactive oxygen intermediates and NO may have several important implications on muscle function, as discussed below.

The effect of exogenous NO on muscle function during hypoxia was studied using the long acting NO donor, DETA NONOate. It is a very stable compound and spontaneously dissociates in solution releasing NO in a controlled and predictable fashion, following first order kinetics. The half-life of NO released by DETA/NO is >500 minutes (17). In order to study the effect of basally released NO on muscle function during hypoxia, we inhibited its production with the NOS inhibitor, L-NAME. L-NAME is a L-arginine analog, which competitively inhibits nitric oxide synthase (NOS), with a relatively greater selectivity of inhibition toward constitutive NOS (13,46).

Finally, to evaluate if the effects of endogenous NO in our study were mediated via its effects on the cGMP pathway, we inhibited this pathway using ODQ, a novel and highly selective inhibitor of soluble guanylyl cyclase. ODQ acts by inhibiting the heme site of the soluble guanylyl cyclase enzyme (42). It does not generate superoxide anions
or inhibit nitric oxide synthase, unlike the two commonly used inhibitors of guanylyl cyclase, LY-83583 and methylene blue (42,45). Our data showed that ODQ treated muscles were comparable to control tissue, thus excluding the effect of endogenous NO via the cGMP pathway, in our model. We also studied the effect of different doses of L-NAME, DETA/NO and ODQ to determine if the effects of NO on muscle function were dose-dependent. We did not however, observe any dose-dependent effect of the drugs studied.

The paradoxical results showing protection of muscle function during hypoxia in the absence of endogenous NO or with excess exogenous NO may be explained by a number of potential mechanisms:

1. The absence or excess of NO could result in an alteration of the NO-\textsuperscript{-}O\textsubscript{2} balance, thereby, decreasing the formation or reactivity of the potent oxidant peroxynitrite and hence, protecting muscle function.

2. \textsuperscript{1}NO, as well as NOS inhibitors could function as antioxidants.

3. Both \textsuperscript{1}NO and L-NAME could influence metabolic pathways which may be critical to muscle function, during hypoxia.

4. NO and L-NAME may have effects on the contractile apparatus and modulate muscle function.

The \textsuperscript{1}NO-\textsuperscript{-}O\textsubscript{2} balance

In biological systems, the highly reactive property of NO is not because of its free radical nature, but due to its reaction with certain other molecules, such as other free
radicals and transition metals (4). The effects of NO are determined by several factors such as the rate and site of production of NO and O₂⁻, relative concentrations of reactive species and antioxidants, as well as the mechanism of oxidative damage during production of NO and O₂⁻ in the tissue (10,41). Thus, NO can function both as a pro-oxidant and as an antioxidant.

The pro-oxidant effects of NO are believed to be largely due to its reaction with O₂⁻ to form a potent oxidant, peroxynitrite. Peroxynitrite is a potent oxidant with "hydroxyl radical-like activity" and has been shown to oxidize thiols, sulfides, lipids and deoxyribose (38). In addition, peroxynitrite has also been shown to cause irreversible inhibition of mitochondrial respiration, perhaps by inhibiting aconitase, an enzyme which is more sensitive to exposure by peroxynitrite and O₂⁻ than NO (58).

Therefore, in our study it is likely that treatment with exogenous NO or removal of endogenous NO resulted in altering the NO-O₂⁻ balance in one direction or the other, thereby, decreasing production of peroxynitrite and, hence resulting in protection of muscle function.

Several studies have underscored the importance of the balance between NO and O₂⁻ (7,31,36,57,58). In their review, Darley-Usmar et al. discussed the role of interaction between reactive oxygen species and reactive nitrogen species in the involvement of several pathological conditions such as cancer, chronic inflammation, Parkinson's disease and atherosclerosis (7). They suggested that a protective strategy under such conditions may be by altering the balance either toward one direction by decreasing NO with NOS inhibitors or decreasing O₂⁻ with the scavenger, superoxide
dismutase or alternatively, toward the other direction by increasing NO using NO donors. Interestingly, in a previous chapter of rat diaphragm function using the same model of hypoxia, we demonstrated protection of muscle function using both intracellular and extracellular antioxidants, such as N-acetylcysteine, DMSO, superoxide dismutase and Tiron (1,2 dihydroxybenzene-3,5-disulfonate) (25). It is conceivable that in these previous experiments, the above antioxidants may have protected muscle function by scavenging free radicals such as $O_2^\cdot$, thereby, decreasing production of peroxynitrite.

\textit{NO and NOS inhibitors as antioxidants}

Several studies have shown that nitric oxide protects cells from cytotoxicity by reactive oxygen species (16,27,56,62), as well as functions as an antioxidant (20). It is believed that NO may play a protective role by preventing formation of oxidants by interacting with metals, scavenging the radicals and oxidants formed, and/or by terminating lipid peroxidation by interaction with lipid radicals (41,54). In addition, recently, White et. al. showed that NO can increase levels of glutathione, an endogenous antioxidant (53). They speculated the mechanism of this increase to be possibly due to increased glutamate (derived from L-arginine), which is a precursor of glutathione.

Likewise, it has been reported that NOS inhibitors may function as antioxidants (18). In their review of a previous study (19), Jessup et. al. point out that, the action of L-N-monomethylarginine (L-NMMA), a NOS inhibitor, in preventing low-density lipoprotein oxidation was independent of its effect on the NOS enzyme (18). In addition, it was recently suggested that neuronal NOS (nNOS) can also produce $O_2^\cdot$, by one
electron reduction of molecular oxygen (57). Interestingly, treatment with the NOS inhibitor, L-NAME was shown to inhibit both O$_2^-$ and NO production by this enzyme (37,57).

Influence on contractile apparatus or metabolic pathways

Increasing evidence suggests that NO influences skeletal muscle contractile function either by directly affecting excitation-contraction coupling, by regulating metabolic pathways by its action on critical enzymes/proteins, or via signal transduction (1,3,9,39,48,51). NO has been shown to bind directly to the calcium release channel (or, ryanodine receptor) by trans-nitrosylation of regulatory thiols on the channel (48). Furthermore, recently, Aghdasi et. al. reported that NO also prevents oxidation of the ryanodine receptor by oxidants by inhibiting cross-linking between the receptor subunits (1). Similar reports of the influence of NO on intracellular calcium has also been shown in cardiac cells (isolated human atrial myocytes), where treatment with the NO/peroxynitrite donor, 3-morpholinosynodiomine (SIN-1) resulted in an increase in the cardiac calcium current (I$_{Ca}$) (23).

It has been proposed that NO can also modulate muscle function by activating second messenger systems via cGMP or other signal transduction systems (24,39,47). Studies by Murrant and Barclay showed that treatment with NO donors resulted in increased production of force in both fast twitch and slow twitch skeletal muscle (29), as well as slowed the rate of fatigue in slow twitch skeletal muscle (30). In the former study, the increase in force in the treated muscles persisted even after washout of drug
and the authors suggested a possible involvement of a long lived second messenger to explain the persistent effects.

Lastly, it is likely that NO may also play an important role in regulating energy metabolism in skeletal muscle cells. Recently, several studies have reported that NO can facilitate glucose uptake in both resting as well as contracting skeletal muscle (2,3,9,40,63). This may be of physiological significance especially in an energy-deficient hypoxic muscle, which may be dependent on glycolysis for ATP production.

There is evidence to suggest that the NOS inhibitor, L-NAME also exerts metabolic effects (8). In their study on isolated rabbit heart, Depre et. al. reported a decrease in ischemic contracture as well as functional protection in hearts treated with 1 μM L-NAME and L-NMMA (8). Using 31P nuclear magnetic resonance spectroscopy, they showed the protective effects to be due to preservation of high energy phosphates in the NOS inhibitor-treated hearts. They also demonstrated stimulation of glycolysis from exogenous glucose in the buffer, thereby, resulting in preservation of endogenous glycogen stores within the muscle.

It is interesting to note that the effect of NO on different organ systems has been shown to be inconsistent between different studies (24,29), (5,6), and (54,55). These discrepancies may be due to several factors including methodological differences such as, type of tissue preparation, dose of drugs used, etc. (6). In addition, it has been reported that differential effects of NO may be observed depending upon the type of drug used (54). For instance, in their studies, Wink et. al. showed that NO donors which release measurable amounts of NO such as, diethylamine nonoate (DEA/NO), S-
nitrosoglutathione (GSNO) and S-nitrosoacetylpenicillamine (SNAP) protected the cells from cytotoxicity while, NO donors which do not release free NO levels (of >200 nM), such as SIN-1 and sodium nitroprusside enhanced the cytotoxicity by hydrogen peroxide (54). Likewise, while both L-NAME and N\(^\circ\)-Nitro-L-arginine (L-NNA) are eNOS inhibitors, it is possible that L-NAME could also produce differential effects since it has been shown to additionally inhibit O\(_2^-\) production by NOS (37).

**Conclusions**

In conclusion, our results demonstrate that during hypoxia, both lowering of endogenous NO, as well as supplementation with exogenous NO is protective to muscle function. Furthermore, it is unlikely that endogenous NO may function via the cGMP pathway since inhibiting production of cGMP via ODQ had no effect on muscle function. The exact mechanism is unclear; however, it may be due to one or perhaps a combination of several different mechanisms, as discussed above.
LIST OF REFERENCES


CHAPTER 5

OXIDATIVE STRESS IN HYPOXIC RAT DIAPHRAGM MUSCLE

INTRODUCTION

Earlier chapters have described the changes in skeletal muscle function during hypoxia and upon reoxygenation as well as the effect of treatment with different antioxidants during these gas-phases. The results indicated a significant decline in twitch and tetanic tensions during hypoxia, with incomplete recovery upon reoxygenation. However, in muscles treated with different intracellular and extracellular antioxidants, there was a significant prevention in the decline in muscle tension during hypoxia as well as an improvement in recovery during reoxygenation.

Since antioxidants improved skeletal muscle function during hypoxia, we hypothesized that during hypoxia there is increased production of oxidants of sufficient magnitude to result in oxidative stress. It follows, therefore that the antioxidant effects on muscle function during
hypoxia reflects their role in protecting critical cell proteins and lipids from oxidation during oxidative stress. In order to test this hypothesis, we used two common measures of oxidative stress to determine if hypoxic muscle cells exhibit evidence of oxidative stress and if antioxidants prevent oxidative stress in hypoxia. The two measurements of oxidative stress used were, the glutathione (GSH) redox status and a measurement of lipid peroxidation using an 8-isoprostane enzyme-linked immunoassay (ELISA).

The glutathione redox system is one of the primary antioxidant systems in the cell and has been widely used as an indicator of oxidative stress (5,7,8,14,46). Normal non-stressed and healthy cells have a high concentration of reduced glutathione (>95% of the total glutathione). However, under conditions of oxidative stress there is a loss of total intracellular GSH and/or an increase in the oxidized form of glutathione (glutathione disulfide or GSSG), resulting in a high GSSG/GSH ratio (11,21,39). A high GSSG/GSH ratio is relevant to function because increases in GSSG can directly result in protein disulfide formation on sulfhydryl groups of a wide variety of cell proteins (13).

The lipid peroxidation product, 8-isoprostane is a non-enzymatically derived prostaglandin (PG)-like intermediate formed from auto-oxidation of arachidonic acid (30). It is considered to be a reliable index of both in vivo and in vitro lipid peroxidation reactions (28). It is present even under normal conditions (28) and has been shown to increase in several models of oxidant stress including animal models of oxidant injury (3,22), as well as in patients with disease processes involving oxidative stress such as, hepatorenal syndrome (33), scleroderma (41), diabetes (15) and also in smokers (29).
The results indicate no evidence of oxidative stress in hypoxic muscles. Although there were small differences in the relative amounts of oxidized glutathione with hypoxia and antioxidant treatment, there was no significant difference in the overall glutathione redox status (total and oxidized glutathione) in hypoxic (exposed to 95% N₂/5% CO₂) muscle compared to normoxic muscle (95% O₂/5% CO₂). Similarly, there was no significant difference in 8-isoprostane levels in hypoxic versus normoxic muscles. Therefore, the results do not support the hypothesis that the mechanism of action of antioxidants in hypoxic diaphragm is via protection from oxidative stress.

METHODS

General Surgical procedure and muscle strip preparation

The experiments were conducted on diaphragm muscle strips taken from adult male Sprague Dawley rats (300-500 g). The animals (n=8 for glutathione assay and, n=3 for 8-isoprostane ELISA) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg), tracheotomized and mechanically ventilated. The phrenic nerve was severed and four diaphragm strips (approximately 0.5 cm) were dissected out with portions of rib and central tendon intact. In addition, a single piece of diaphragm from the contralateral side was freeze-clamped in liquid nitrogen for later analysis of GSH redox status or 8-isoprostane levels from what was considered “fresh” normal in situ diaphragm.
The strips were mounted vertically with the central tendon positioned superiorly and attached to a force transducer which was connected to a micropositioner. D-tubocurarine (10µM) was added to eliminate activation of intramuscular nerve branches. The diaphragm strips were positioned between two platinum plates and stimulated by direct electrical field.

The tissues were placed in four different tissue baths containing physiological salt solution (in mEq/L: 21 NaHCO₃, 0.9 NaSO₄, 1.2 NaHPO₄, 0.9 MgCl₂, 2.25 CaCl₂, 5.9 KCl, and 109 NaCl and 2.07 g/L glucose). The baths were maintained at 37°C and were continuously bubbled with 95% O₂-5% CO₂ during normoxia and 95% N₂/5% CO₂ during the hypoxic phase. Two of the tissue-baths were treated with the antioxidant, NAC (18 mM). All tissues were exposed to an initial normoxic gas mixture and muscle function (twitch characteristics and stress-frequency relationships) was measured. Then, two of the tissue baths (one treated with NAC and the other untreated) were exposed to 30 minutes of hypoxia and muscle function was measured again, following which, all four muscles were immediately freeze-clamped with metal tongs equilibrated with liquid nitrogen. The hypoxic tissues did not undergo reoxygenation prior to freeze clamping. The frozen tissues were stored at -80°C, until further analysis.

1. Determination of Muscle glutathione levels

The four different tissues (n=8, in each group) obtained were as follows: control tissue in normoxia, NAC-treated tissue in normoxia, control tissue in hypoxia, and NAC-treated tissue in hypoxia. Tissues were stored at -80°C overnight and the 5,5'-dithiobis(2-nitrobenzoic acid)/GSSG reductase recycling glutathione assay (1) was performed the next
Comparisons were made with additional tissue obtained from fresh muscle, directly freeze-clamped while in-situ.

The muscle strips were homogenized in 5% sulfosalicylic acid (in order to stop further oxidation of GSH to GSSG), to produce a 20% homogenate. Homogenization was done using 1 mm glass beads in a mini bead-beater (Biospec products, Bartlesville, OK), with intermittent cooling during the process. The homogenates were centrifuged at 10,000 rpm for 5 minutes. The glutathione assay was performed on the supernatants. The total glutathione content in the muscle was estimated using a modified enzymatic recycling assay of Griffith (16), originally adapted for use in diaphragm samples by Borzone et. al. (7). In this method, glutathione, is sequentially oxidized by 5,5'-dithiobis,2-nitrobenzoic acid (DTNB) and reduced by NADPH in the presence of glutathione reductase. The rate and amount of 2-nitro-5-thiobenzoic acid is then determined spectrophotometrically at 412 nm to give the amount of glutathione present in the sample when compared to the standard curve (16).

In order to determine GSSG, the samples were derivatized using 2-vinylpyridine (16). About 6 μl of 2-vinylpyridine and 1 μl of triethanolamine (to adjust pH) was added to about 300 μl of the homogenate supernatant. The derivatization process binds all the reduced glutathione (GSH) and prevents it from participating in the enzymatic reactions. The samples were then assayed as described above for GSH (16).

Each day, separate standards were run both for reduced (GSH) and oxidized glutathione (GSSG). All samples were run in triplicate. Total glutathione (GSH₅ = GSH + GSSG) is expressed as nanomoles per gram wet weight. For the ratio of GSSG/GSH₅, GSSG is expressed as nanomoles of glutathione equivalents per gram wet weight of the tissue. The
term glutathione equivalents is necessary since two GSH molecules make up the disulfide form (GSSG). Therefore, to calculate the % of GSH which is in the GSSG form, \((2 \times \text{GSSG})/\text{GSH}_r\) is commonly used for the fraction of GSH molecules in the oxidized state.

2. Determination of 8-isoprostane in hypoxic diaphragm muscle

In order to test if hypoxia causes oxidative stress resulting in lipid peroxidation, we measured total \(F_2\)-isoprostanes in control and hypoxic rat diaphragm muscle strips, with and without treatment with NAC \((n = 3, \text{ in each group})\) using a competitive enzyme-linked immunoassay. We also measured \(F_2\)-isoprostanes in \textit{in situ} freeze-clamped rat diaphragm.

The procedure used to extract \(F_2\)-isoprostanes is a modified protocol as described by Morrow and Roberts (32). Each frozen muscle strip was homogenized using mortar and pestle under liquid nitrogen. The homogenized sample was transferred to an eppendorf and 1 ml ethanol containing 0.005% butylated hydroxy toluene (BHT) was added to it and vortexed. BHT is a free radical scavenger and was added to the tissue to prevent \textit{ex vivo} lipid peroxidation and the formation of isoprostanes (32). The sample was then cooled on ice for \(\sim 5\) minutes, followed by centrifugation at 1500 g for 10 minutes, in order to precipitate proteins. The supernatant was carefully decanted into a test-tube. An equal volume of 15% potassium hydroxide was added to the supernatant and the mixture was incubated at 40°C for one hour, to enable alkaline hydrolysis of the esterified isoprostanes. Following incubation, the volume of the sample was brought up to 10 ml by addition of ultrapure water and its pH was adjusted to \(\sim 3.0\) with addition of hydrochloric acid.
The lipid in the above sample was then extracted by passing the sample through a column of C18 Sep-Pak, and subsequently through a column of silica Sep-Pak. The sample was finally eluted in ethyl-acetate:methanol (1:1), from the silica Sep-Pak. The eluate was evaporated under a steady stream of dry nitrogen.

The extracts thus obtained are a measure of total (free + esterified to phospholipids) F2-isoprostanes. The extracts were reconstituted into multiple dilutions and assayed by enzyme-linked immunoassay (using 8EPGF2 immunoassay kit, Oxford Biomedical Research). F2-isoprostane in the samples were quantified using a separate standard curve performed with each assay. The values are expressed in nanograms of F2-isoprostanes per gram wet weight of tissue.

Data analysis

All data are expressed as group means ± SEM. Glutathione data was analyzed using a two-way ANOVA with treatment and gas phase being independent variables and various glutathione measures being dependent variables. Due to small “n=3”, 8-isoprostane data were analyzed using student’s t test. A P < 0.05 was considered to be statistically significant.
RESULTS

Muscle function data

The influence of hypoxia and antioxidants on measures of muscle function during hypoxia (not shown) were similar to those described under chapter 2. In brief, during hypoxia, there was a marked fall in twitch and tetanic tensions. However, the decline in muscle function during hypoxia was less in muscles treated with NAC. Therefore, NAC showed a protective effect on muscle function during hypoxia, as described in Chapter 2.

Muscle glutathione data (Table 11 and Fig. 31)

Total glutathione (GSH\(_T\) = GSH + GSSG): There was approximately a 50% reduction in total glutathione in diaphragm strips maintained within the tissue bath as compared to diaphragms freeze-clamped directly from the living animal (values, bottom of Table 11). Measures of total glutathione in fresh in situ diaphragm were similar to previous reports (7). There was also a significant increase in GSH\(_T\) in NAC treated normoxic tissues compared to untreated normoxic tissues \((P < 0.01)\). No other significant changes in total glutathione between groups were observed.

Oxidized glutathione (GSSG): In the untreated tissues there was a small but significant increase in GSSG during hypoxia \((P < 0.05)\). Both NAC treated tissues had significantly lower GSSG compared to the untreated tissues \((P < 0.01)\) and, in fact, approached zero.
Control (untreated)       NAC-treated

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSHₜ</td>
<td>684 ± 83</td>
<td>786 ± 47</td>
<td>860 ± 62‡</td>
<td>776 ± 54</td>
</tr>
<tr>
<td>GSSG</td>
<td>11 ± 2.0</td>
<td>17 ± 3.0*</td>
<td>3.0 ± 2.0‡</td>
<td>0.0 ± 0‡</td>
</tr>
<tr>
<td>GSSG%</td>
<td>3.4 ± 1.0</td>
<td>4.4 ± 0.8</td>
<td>0.8 ± 0.5§</td>
<td>0.0 ± 0§</td>
</tr>
</tbody>
</table>

Table 11. *Muscle glutathione.*

All data are expressed as means ± SEM. Data shown represents total glutathione (GSHₜ nmol/gm wet wt.), oxidized glutathione (GSSG nmol/gm wet wt.) and % oxidized glutathione (in GSSG equivalents/GSHₜ).

Freeze-clamped tissue (in situ): GSHₜ = 1356 ± 65, GSSG% = 6.1 ± 0.6%. Post hoc contrasts following ANOVA, *P < 0.05, § P < 0.01, ‡P < 0.005: differences from normoxic control.
Figure 31. Muscle glutathione levels in untreated tissue and tissue treated with NAC. *Left bars:* There was no change in total glutathione levels during hypoxia in the treated and untreated tissues. *Center and right bars:* There was a significant increase in oxidized glutathione (GSSG), but no change in the glutathione redox poise (2GSSG/GSHt%) of the muscle during hypoxia. NAC treatment significantly reduced GSSG levels during hypoxia.
Figure 31.
There was no significant difference between GSSG in the NAC treated tissues during hypoxia vs. normoxia. The fresh, *in situ* diaphragm exhibited elevated levels of oxidized glutathione (Table 11, legend).

**Oxidized Glutathione % [(2GSSG/GSH<sub>T</sub> )<sub>100 %]**: All NAC treated tissues had significantly lower fractions of oxidized glutathione to total glutathione when compared to untreated (control) tissues ($P < 0.005$). However, there was no significant increase in the fraction of oxidized glutathione in the untreated tissues during hypoxia vs. normoxia ($P = 0.22$). Interestingly, the ratio was increased in *in situ* tissue compared to tissue exposed to the tissue bath ($P < 0.05$).

**8-isoprostane enzyme immunoassay**

There was no difference in total 8-isoprostane levels between the four different groups of muscles: normoxic and hypoxic muscles with and without treatment, Table 12. Thus, there was no evidence of lipid peroxidation during hypoxia, as compared to normoxia. Furthermore, there was no difference in 8-isoprostane levels between *in situ* frozen muscles and muscles equilibrated in tissue baths.

**DISCUSSION**

There was no clear evidence of oxidative stress in hypoxic rat diaphragm muscles. Although, the oxidized glutathione (GSSG) was significantly increased in hypoxia, there was no significant increase GSSG/GSH<sub>T</sub> ratio under these conditions. Therefore, there was no
Table 12. Levels of total 8-isoprostane in *in situ muscle*, untreated normoxic muscle, untreated hypoxic muscle, NAC-treated normoxic muscle and NAC-treated hypoxic muscle. Each 5 rows represents results from one animal.
<table>
<thead>
<tr>
<th>MUSCLE</th>
<th>8-isoprostane (nmol/g wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh frozen</td>
<td>121</td>
</tr>
<tr>
<td>Control, Normoxia</td>
<td>299</td>
</tr>
<tr>
<td>Control, Hypoxia</td>
<td>173</td>
</tr>
<tr>
<td>NAC, Normoxia</td>
<td>604</td>
</tr>
<tr>
<td>NAC, Hypoxia</td>
<td>780</td>
</tr>
<tr>
<td>Fresh frozen</td>
<td>242</td>
</tr>
<tr>
<td>Control, Normoxia</td>
<td>242</td>
</tr>
<tr>
<td>Control, Hypoxia</td>
<td>135</td>
</tr>
<tr>
<td>NAC, Normoxia</td>
<td>140</td>
</tr>
<tr>
<td>NAC, Hypoxia</td>
<td>137</td>
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<tr>
<td>Fresh frozen</td>
<td>271</td>
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<tr>
<td>Control, Normoxia</td>
<td>231</td>
</tr>
<tr>
<td>Control, Hypoxia</td>
<td>213</td>
</tr>
<tr>
<td>NAC, Normoxia</td>
<td>319</td>
</tr>
<tr>
<td>NAC, Hypoxia</td>
<td>366</td>
</tr>
</tbody>
</table>

Table 12.
alteration in glutathione redox poise in hypoxic muscles. Similarly, on analysis for lipid peroxidation products, there was no increase in 8-isoprostane levels in hypoxic muscles. These findings suggest that while oxidizing species generated during hypoxia are responsive to a wide range of antioxidants (27), they do not appear to influence, or are of insufficient magnitude to result in significant changes in glutathione redox state or to induce significant lipid peroxidation.

**Glutathione redox system**

Glutathione (γ-glutamyl-cysteinyl-glycine) is an abundantly present low molecular weight tripeptide in the body (11), Fig 32. It is present in high concentration intracellularly (0.5-10 mM) and relatively lower concentration extracellularly (in μM) (24). The concentration of glutathione in skeletal muscle is ~ 1 mM (17,20). The glutathione redox system has been implicated in many cellular functions and is also one of the most important cellular antioxidants (25). This system is composed of two tightly coupled enzymes namely, glutathione peroxidase and glutathione reductase which regulate GSH (reduced glutathione) and GSSG (oxidized glutathione) levels in the cell (Fig 33). These enzymes are found mainly in the cytosol, although some glutathione redox activity is also found in the mitochondria (46). The antioxidant property of glutathione is because of its thiol group and GSH is the main intracellular thiol (39). It therefore functions as an intracellular reductant and reduces oxidants such as hydrogen peroxide and other peroxides (25). Glutathione is also a free
Figure 32. Structure of glutathione. Glutathione is a low molecular weight tripeptide (γ-glutamyl-cysteinyl-glycine). Molecular weight = 307.3
Figure 32.
Figure 33. The glutathione redox system.
Figure 33.
radical scavenger of hydroxyl radical and singlet oxygen. In addition, it is known to be involved in several important cell functions such as DNA and protein synthesis, transport and metabolism (11,17,23,26).

Glutathione is synthesized via the γ-glutamyl cycle from glutamic acid, cysteine and glycine catalyzed by glutathione synthetase and by the rate limiting enzyme γ-glutamylcysteine synthetase reaction, (11). Although glutathione exists in low concentration in the extracellular environment, most cells do not have the capacity to transport it; rather, it is synthesized from its constitutive parts, cysteine and glycine (24). It is believed that the transportation of amino-acids required for glutathione synthesis is well regulated by a membrane bound γ-glutamyl transpeptidase (24,26).

A lowering of GSH to 20-30% of its physiological levels may result in impaired cellular defense, cell injury and even cell death (39). Under normal conditions, GSSG is easily reduced back to GSH via the glutathione reductase enzyme using NADPH as the reductant. Thus, NADPH and glutathione reductase can replete GSH levels (see Fig. 3). However, under conditions of oxidative stress, it may be possible for the excess GSSG levels to overwhelm glutathione reductase capacities (39). Consequently, a build-up of intracellular GSSG can occur which is toxic to the cell and can be actively exported out of the cell (11,39).

Increased oxidized glutathione has also been demonstrated in several studies on skeletal muscle under conditions of intense muscle activity, such as in inspiratory resistive loading of the diaphragm and intense exercise in limb muscles (2,10,20,44). The increase in GSSG in these studies has been attributed to oxidative stress. Based on our previous study where antioxidants significantly prevented a decline in muscle function during hypoxia (27),
we hypothesized that hypoxia is characterized by oxidative stress and, therefore, may show changes in glutathione redox poise in the cell. We saw a small but significant increase in the GSSG levels in the untreated muscles subjected to hypoxia vs. their normoxic controls. However, despite the rise in GSSG, there was no significant change in GSSG/GSH\textsubscript{r} ratio during hypoxia. This may be explained by any of the following speculations: 1) The NADPH concentration in muscle has been shown to increase following hypoxia (18,40). Thus, changes in redox ratio may not have been observed due to the rapid recycling of oxidized glutathione back to reduced glutathione, utilizing the increased NADPH. 2) oxidative insult was not large enough to produce discernable changes in the glutathione redox ratio. 3) Some GSSG was actively shuttled out of the cell (39). This, however, was not evident from changes in GSH\textsubscript{r} in hypoxic muscle.

NAC is a thiol containing antioxidant and has been shown to replenish glutathione levels in the cell by providing cysteine, a limiting substrate for GSH synthesis (39). As expected, there were significant increases in GSH\textsubscript{r} levels in the non-hypoxic NAC-treated muscles but interestingly, no increase in GSH\textsubscript{r} in NAC treated hypoxic muscles. This suggests that hypoxia may have interfered with the ability to utilize NAC as a substrate for GSH synthesis. A marked reduction in GSSG was observed in all NAC treated tissues. In fact, GSSG was driven down to levels in most tissues which were below the level of sensitivity of the enzymatic assay.

One possible scenario that could explain the influence of NAC during hypoxia is that critical proteins which influence force production in hypoxia may be under tight regulation of the normal redox state of the cell. Their activity may be greatly elevated during hypoxia
and therefore be very sensitive to apparent "minor" changes in redox poise. For example, enzymes responsible for maintenance of energy status of the cell during hypoxia via glycolysis are greatly up-regulated in hypoxia and are known to be highly redox sensitive with critical cysteines at their reactive site (34). Lowering GSSG to zero may therefore drive these enzymes to a more reactive chemical state. This scenario is highly speculative but none the less conceivable. Furthermore, calcium sensitivity of skinned cardiac muscle fibers has been shown to be sensitive to large changes in glutathione redox poise (6). Whether smaller changes in redox poise can affect calcium sensitivity is not clear.

Interestingly, there was also an ~ 50% reduction in total glutathione levels in muscles in the tissue baths, as compared to in situ freeze-clamped muscle. There are a number of possible implications. For example this may represent a leakage of glutathione from muscle strips into the tissue bath solution due to damage to cell membranes or it may reflect active transport of GSSG out of cells in response to an elevated oxidative stress imposed by the tissue bath alone. The high oxygen in the bath prior to hypoxia may induce its own form of oxidative stress to the cells.

8-isoprostane enzyme linked immunoassay

In biological systems, lipids are considered to be the most vulnerable to free radical attack, compared to all the other biomolecules. This may be largely due to self-perpetuating chain reactions propagated by the formed lipid radicals (9). In 1990, Morrow et. al., in a stored sample of plasma, discovered a class of non-enzymatically derived prostaglandin (PG)-
like intermediates formed from auto-oxidation of arachidonic acid, which further underwent reduction to finally result in a series of PGF₂-like compounds (30).

The latter, now collectively known as F₂-isoprostanes (containing F-type prostane rings), are considered to be a reliable index of both in vivo and in vitro lipid peroxidation reactions (28). This assay has become the standard in the field of free radical biology (38). F₂-isoprostanes, present even under normal conditions, have been shown to increase in several animal models of oxidant stress, as well as in patients with disease processes involving oxidative stress (28). Furthermore, these compounds have been shown to manifest biological activity such as pulmonary and renal vasoconstriction (4,31), induction of vascular smooth muscle cell mitogenesis (45), and may themselves cause oxidant injury (28).

F₂-isoprostanes exist in two forms - free and esterified. The latter is found esterified to phospholipids and may be released in its free form by the action of phospholipase or by alkaline hydrolysis (32). Thus, measurement of both free and esterified forms gives an accurate measurement of total F₂-isoprostanes. This relatively new method of assaying F₂-isoprostanes offers almost 100% specificity and is simple to perform compared to the conventional time consuming and expensive method using mass spectrometry (32).

To the best of our knowledge, this is the first study to quantify total isoprostane in rat diaphragm under any conditions. Previous studies have measured only free F₂-isoprostanes in rat (47) and hamster (42) diaphragms under normal conditions and in oxidative stress. Similar to results on glutathione redox system, we did not see any evidence of increased lipid peroxidation products in the hypoxic muscles. It is important to note, however, that the 'n' for these experiments was extremely small, making conclusions from these experiments highly
speculative. Further experiments will be needed to verify these preliminary results for F₂-isoprostanes.

Based on these results, it may be concluded that in our model of hypoxia, although antioxidants significantly protect muscle function during hypoxia, the decline in muscle function during hypoxia is unlikely to be due to overwhelming oxidative stress. Potential injury or damage could explain the ~ 70% recovery of muscle function seen following reoxygenation (27). It is also possible that reactive oxygen species generated during hypoxia influence muscle function by other mechanisms such as alteration of ionic balance within the cell, affecting critical metabolic pathways or enzyme activity, minor changes in redox status of the cell, etc.

In recent years, it has become increasingly recognized that most cells are producing and regulating reactive oxygen species under normal conditions. This certainly is true of skeletal muscles in which both intra- and extracellular ROS production has been demonstrated in "resting" muscle (36,37). Furthermore, reactive oxygen has been shown to be an intracellular signaling pathway by which many cytosolic and nuclear control systems are modulated (19). The results of these experiments suggest the possibility that ROS may be functioning as a cell regulator rather than as an inducer of oxidative stress. The next hypothesis to be tested is that antioxidants functioned in hypoxia by modulation of a ROS pathway of cell regulation which served to down regulate function in hypoxia, perhaps in an attempt to protect the cell from injury, which could occur during activation in hypoxic or anoxic conditions. Such a potential role of oxidants as a participant in the fatigue process has previously been proposed (7,12,13,35,43).
Conclusions

There was significant decline in skeletal muscle function during hypoxia which was markedly attenuated in muscles treated with NAC. However, there was no clear evidence of oxidative stress in the hypoxic muscle as evidenced by the unaltered glutathione redox poise and the absence of lipid peroxidation products. Therefore this model of hypoxic diaphragm, the oxidant generation is large enough to cause significant decline in muscle function; however, it is not of sufficient magnitude to cause significant oxidative stress.
LIST OF REFERENCES


CONCLUSIONS

This study was performed to assess the influence of reactive metabolites of oxygen and nitrogen on diaphragm muscle function during hypoxia. Studies on cardiac muscle have shown that hypoxia results in both oxidant formation and oxidative stress which are not further exacerbated by subsequent reoxygenation (15,16). The mechanism responsible for oxidant production in hypoxia is not well understood and has not previously been demonstrated in skeletal muscle. The study of hypoxic skeletal muscle has several important clinical implications such as, under conditions of respiratory failure and associated cardiovascular collapse and resuscitation, as well as in organ transplants (finger), where conditions are appropriate for local tissue hypoxia and ischemia-reperfusion to occur.

The experiments were based on the hypothesis that reactive species of oxygen and nitrogen depress rat diaphragm muscle function during hypoxia and contribute to the decline in muscle function seen during hypoxia. Both intracellular and extracellular antioxidants were administered and they significantly inhibited the decline in muscle function during hypoxia as well as improved recovery upon reoxygenation. To assess the role of reactive nitrogen species on muscle function in hypoxia, the effect of i) inhibiting endogenous nitric oxide (NO)
with nitric oxide synthase (NOS) inhibitor, L-NAME, ii) increasing exogenous NO with NO donor, DETA/NO and, iii) blocking the effect of endogenous NO via cGMP pathway with ODQ were studied. Paradoxically, muscle function during hypoxia was protected during both, inhibition of endogenous NO as well as with excess exogenous NO. The effects of endogenous NO were however independent of the cGMP pathway.

Although antioxidants and NO modulators protected muscle function during hypoxia, the protection was incomplete (~30% protection with antioxidants) and also function did not recover completely. Therefore, during hypoxia suppression of muscle function is likely to be caused by other factors in addition to reactive species of oxygen and nitrogen. These may include, 1) excess intracellular calcium levels due to inhibition of plasma membrane ATPase and Na'/Ca²⁺ exchanger (5). The excess calcium ions can activate proteases which destroy the sarcolemma and cytoskeleton as well as cause breakdown of muscle membrane phospholipids by the action of calcium-activated phospholipases (9). Therefore, function may be reduced by calcium-induced cell injury. 2) changes in the intracellular milieu occur under conditions of hypoxia including a rise in inorganic phosphate, decline in pH, a fall in phosphocreatine levels and changes in the concentrations of adenine nucleotides. These changes can directly depress the contractile apparatus, causing a decline in muscle function (8). 3) changes in membrane permeability during hypoxia can result in ionic imbalances within the cell, thus impairing cell function. It is possible that one or all of these factors, in combination with reactive species, may contribute to depression of muscle function during hypoxia.
In general, the effects of NO modulators on muscle function during hypoxia were minimal compared to that of antioxidants. Therefore, it is possible that NO/reactive nitrogen species are not a major player in influencing muscle function during hypoxia. The paradoxical results showing protection of muscle function during hypoxia in the absence of endogenous NO or with excess exogenous NO may be explained by a number of potential mechanisms, 1) The absence or excess of NO can result in an alteration of the NO- \( \text{O}_2^- \) balance, thereby, decreasing the formation or reactivity of the potent oxidant peroxynitrite and hence, protecting muscle function (4). Previous studies have shown that NO, as well as NOS inhibitors can function as antioxidants (10,11). 3) Both NO and L-NAME may influence metabolic pathways which may be critical to muscle function, during hypoxia (3,7,18). 4) NO and L-NAME may have effects on the contractile apparatus and modulate muscle function (21).

Finally, the presence of oxidative stress during hypoxia was assessed by measuring glutathione redox state and lipid peroxidation products in the hypoxic muscles. There was no evidence of oxidative stress in the hypoxic muscle as evidenced by the unaltered glutathione redox poise and absence of lipid peroxidation products.

It may be concluded from the above results that in this model of hypoxic skeletal muscle, while reactive oxygen and nitrogen species influence muscle function during hypoxia, it is possible that they modulate muscle function without causing irreversible oxidative injury or damage to the muscle. For instance, it may be speculated that reactive species of oxygen and nitrogen modulate muscle function by influencing critical enzyme/metabolic pathways necessary for muscle function. Several studies have reported that NO can facilitate glucose
uptake in both resting as well as contracting skeletal muscle (2,3,7,19,23) which may be of physiological significance especially in an energy-deficient hypoxic muscle.

Alternatively, reactive species may trigger second messenger pathways, which may in turn modulate cell function. NO has been shown to modulate muscle function by activating second messenger systems via cGMP or other signal transduction systems (13,18,20). Similarly, O$_2^-$ has been shown to activate transcription factors such as NFkB (22). In addition, several other genes important for cell growth and differentiation such as, c-fos, c-myc, c-jun and $\beta$-actin have been shown to be induced by reactive oxygen species (22).

In conclusion, the results of this thesis present an interesting scenario where reactive species may actually play a protective role during hypoxia by down-regulating cell function in a hypoxic tissue. Therefore, it is possible that during potentially harmful conditions to the cell such as, exposure to low oxygen or hypoxic environment, the cell may generate reactive metabolites of oxygen and nitrogen in order to temporarily shut-down cell function, thereby preserving cell viability.

**Benefits and limitations of the model**

Rat diaphragm serves as an excellent model to study hypoxic skeletal muscle function because of its small size and minimal thickness (1-1.5 mm). Thus, when placed within a tissue bath, the core of the muscle is less vulnerable to become hypoxic because of the small diffusion distance. Furthermore, study of diaphragm muscle during hypoxia has several important clinical implications such as during conditions of respiratory failure with associated
cardiovascular collapse and muscle fatigue. Interestingly, several common changes in the intracellular milieu such as, a rise in inorganic phosphate, decline in pH, a fall in phosphocreatine levels and changes in the concentrations of adenine nucleotides occur under conditions of hypoxia or fatigue of a muscle (8).

There are some limitations to this model of hypoxic skeletal muscle. One of them includes the severity of hypoxia/relative anoxia. The gas-mixture concentrations were chosen based on results of preliminary experiments (not shown) which showed that rat diaphragm is relative resistant to low oxygen concentrations. In these experiments, upon repeated twitch stimuli with decreasing oxygen concentrations, a fall in muscle tension was observed only after exposure to anoxia (95% \( \text{N}_2 \), 5% \( \text{CO}_2 \)) for about 2-3 minutes. Thus, all hypoxic experiments were conducted at this gas concentration. Furthermore, several other studies have employed these gas concentrations for studies in hypoxia (12,15,17). The other limitation is the fiber-type composition of rat diaphragm. Rat diaphragm is a skeletal muscle with mixed fiber types and is composed of 40% type I (slow oxidative), 26% type IIA (fast-oxidative-glycolytic/fatigue-resistant), and 34% type IIB (fast-glycolytic/fatigable) fibers (6,14). Thus, the study of function of individual fibers types during hypoxia is not possible using rat diaphragm. Finally, these studies were done on non-perfused muscles in tissue bath. Thus, the role of systemic interaction on muscle function during hypoxia cannot be studied using this isolated system. However, this may be advantageous in the study of nitric oxide (NO) and reactive metabolites of nitrogen on muscle function since the confounding effects of (NO) on circulation is removed.
Future directions

Future studies confirming the conclusions of the different experiments in this thesis may include:

1. Demonstration of the presence of reactive species in hypoxic muscle using fluorescent reactive species probe, hydroethidine. The latter, in the presence of reactive oxygen species is oxidized to ethidium which is also fluorescent, at a different wavelength from hydroethidine. It combines with deoxyribonucleic acid (DNA) in the nucleus and thus remains trapped intracellularly (1).

2. Measurement of high energy phosphates within the hypoxic muscle in the absence and presence of antioxidants in order to determine if antioxidants improve muscle function by altering energy levels within the cell.

3. Detection of the presence of peroxynitrite in hypoxic muscle using nitrotyrosine immunofluorescence in order to determine role of \( \cdot \text{NO-O}_2^- \) balance in hypoxic muscle. Nitrotyrosine is formed by reaction of peroxynitrite with proteins (tyrosine) in the cell. Presence of nitrotyrosine would emphasize the combined presence of reactive metabolites of oxygen and nitrogen and also offer explanation of the paradoxical results in nitric oxide experiments (chapter 4).
LIST OF REFERENCES


APPENDIX

DESCRIPTION OF EXPERIMENTAL PROTOCOL

Muscle preparation

All experiments were conducted on diaphragm muscle strips taken from adult male Sprague Dawley rats (300-500 g). Rat diaphragm is a skeletal muscle with mixed fiber types. It is composed of 40% type I (slow oxidative), 26% type IIA (fast-oxidative-glycolytic/fatigue-resistant), and 34% type IIB (fast-glycolytic/fatigable) fibers (2,3). The fiber types are relatively well mixed in the diaphragm, with a very minimal preponderance of type I fibers on the thoracic compared to the abdominal surface (3).

The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg), tracheotomized and mechanically ventilated. Following thoracotomy, the left phrenic nerve was exposed and severed. About 4 muscle strips (2 cm x 0.5 cm x 1 mm) were excised randomly from left and right hemi-diaphragms, with portions of the last two ribs and central tendon intact (fig. 34). The muscle strips were placed in physiological salt solution (in mEq/L: 21 NaHCO₃, 0.9 Na₂SO₄, 1.2 NaHPO₄, 0.9 MgCl₂, 2.25 CaCl₂, 5.9 KCl and 109 NaCl and 2.07 g/L glucose), bubbled with 95% O₂, 5% CO₂. The tissues were
Figure 34. Diaphragm muscle strips
then randomly designated as experimental or control and placed in tissue baths at 37 °C, containing the same physiological salt solution.

**Stimulation protocol**

The strips were mounted vertically with the central tendon positioned superiorly and attached to a force transducer which was connected to a micropositioner. D-tubocurarine, (10μM), a non-depolarizing neuromuscular blocking agent, was added to eliminate activation of intramuscular nerve branches. This drug binds with nicotinic receptors and prevents depolarization of muscle cell membrane, thus inhibiting contraction via acetylcholine (4). The diaphragm was stimulated to contract by placing it between two platinum electrodes and stimulating it directly in an electric field.

The length of muscle strip was initially adjusted to a resting preload, passive tension of 2 g. The muscle was stimulated electrically to twitch at 0.2 Hz, with gradual increments in current until maximum stimulation current was obtained. This stimulation frequency was shown in preliminary experiments to result in no fatigue of the strips. The maximum current used was 10% above that required to obtain peak twitch tension. At the maximum current, the length of the muscle was then altered by adjusting the micropositioner and $L_0$ was determined as the lowest optimal (minimal) length at which maximum active twitch tension was obtained. Thereafter, all stimulations were performed at $L_0$ and at maximal current. In preliminary unpublished experiments, $L_0$ with tetanic (100 Hz) stimuli was generally obtained at a lower resting length (near zero preload) compared to $L_0$ with twitch stimuli. In all experiments in this thesis, $L_0$ was measured at the minimal length (i.e., at minimal preload) at
which maximal peak twitch tension was obtained, approximately 0.5-3.0 g. In this way, a
"near maximum" zenith of force length curve was attained for tetanic contractions which also
served as an appropriate for $L_0$ for maximum force for twitch contractions.

The contractile function of the muscle was assessed by recording a single twitch
tension (0.5 Hz stimulation) and the peak twitch tension, time to peak tension, and half-
relaxation time were calculated. This was followed by measurement of force at different
tetanic stimulation frequencies (20, 30, 40, 50, 60, 80 and 100 Hz), with an interval of ~30
seconds between each tetanic stimulation at train duration of 400 ms.

**Experimental design**

In general most experiments had one control (or untreated) tissue and three
experimental (or treated) tissues. All four tissues were subjected to the same gas phases, at
the end of which muscle function (twitch and tetanic stimuli) was studied. The different gas
phases were:

a) initial/baseline phase - in normoxia (95% O$_2$, 5% CO$_2$), before treatment with any drug.
b) prehypoxic phase - in normoxia, during ½ Hr. of treatment with drug for the experimental
tissues.
c) hypoxic phase - in hypoxia (95 % N$_2$, 5% CO$_2$) and continued treatment with drug.
d) reoxygenation/recovery phase - in normoxia and continued treatment with drug.
To determine the kinetics of gas pressures in the tissue baths, the Po$_2$ in the baths was continuously monitored with a Clark electrode (BMS3 MK2 Blood Micro System, Radiometer, America Inc., Cleveland, OH). During hypoxia, the Po$_2$ generally dropped rapidly in the tissue baths, reaching <50 mmHg within 3 minutes. In general, a minimum partial pressure, indistinguishable from zero, was reached within 15-20 minutes into the hypoxic period (chapter 2, Fig. 3).

In order to study the integrity of untreated tissue (with respect to muscle function) over time in our model, normoxia time controls were performed. In a previous experiment in our laboratory, we demonstrated that muscle force in a tissue bath in normoxia deteriorates approximately 10 - 20% per hour (Figure 35, Table 13) (1).
<table>
<thead>
<tr>
<th>Muscle function</th>
<th>time 0</th>
<th>½ hour</th>
<th>1 hour</th>
<th>1½ hours</th>
<th>2 hours</th>
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<tr>
<td>Twitch (0.5 Hz)</td>
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<td>4.85</td>
<td>4.29</td>
<td>3.54</td>
<td>3.03</td>
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<td>9.60</td>
<td>8.34</td>
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<td>Tetanus (40 Hz)</td>
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<td>11.11</td>
<td>10.10</td>
<td>9.35</td>
<td>7.58</td>
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<tr>
<td>Tetanus (50 Hz)</td>
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<td>14.65</td>
<td>12.38</td>
<td>10.36</td>
<td>9.60</td>
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<tr>
<td>Tetanus (60 Hz)</td>
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<td>17.43</td>
<td>14.90</td>
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<td>Tetanus (80 Hz)</td>
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<td>16.17</td>
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<tr>
<td>Tetanus (100 Hz)</td>
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<td>21.22</td>
<td>18.69</td>
<td>16.17</td>
<td>14.65</td>
</tr>
</tbody>
</table>

Table 13. Muscle function in untreated tissue over time
Figure 35. Time course of changes in twitch force and tetanic force (50 Hz and 100 Hz) in control muscles during normoxia, stimulated every ½ hour.

A. Twitch force  B. Tetanic force (50 Hz)  C. Tetanic force (100 Hz)

(Note: The tetanic force data represented here have been reduced to 70% original size.)
Figure 35.
LIST OF REFERENCES


BIBLIOGRAPHY


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