MOLECULAR MECHANISMS OF STRESS-INDUCED REACTIVE OXYGEN SPECIES FORMATION IN SKELETAL MUSCLE

DISSERTATION

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By

Li Zuo, B.S., M.S.

The Ohio State University

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Dissertation committee:

Dr. Thomas L. Clanton, Adviser
Dr. Elizabeth L. Gross
Dr. A. John Merola
Dr. Peter J. Reiser

Approved by

Adviser
Biophysics Graduate Program
ABSTRACT

Reactive oxygen species (ROS) play an important role in many biological systems. Skeletal muscles have been shown to generate considerable ROS in resting and in contracting conditions. In this study, I tested the hypothesis that increased ROS production in skeletal muscles is also associated with exposure to two other conditions of stress which are common in normal skeletal muscle during exercise, namely heat stress and hypoxia.

There is no previous direct evidence that ROS are produced during these stresses, particularly in skeletal muscle, but they may play important roles in normal contractile and cell signaling responses. Two assays for superoxide ($O_2^-$) formation were used in rodent diaphragm, the cytochrome c assay for extracellular $O_2^-$ release and the hydroethidine oxidation for intracellular $O_2^-$ formation. The results demonstrate the following: 1) Markedly increased intra- and extracellular ROS formation was observed, particularly $O_2^-$, at temperatures known to be physiologically relevant to exercise (i.e. $42^\circ$C). 2) The process of $O_2^-$ release (extracellular formation) is not directly related to mitochondria, NADPH oxidase, or anion channels, though these are normally believed to be involved in either $O_2^-$ generation or the exit pathway of $O_2^-$ through membranes. 3) Upstream pathways of arachidonic acid (AA) metabolism, both phospholipase A2 and
nitric oxide synthase are associated with $O_2^-$ release. 4) Downstream pathways of AA metabolism are also involved. Though blockage of either cyclooxygenase or cytochrome P450-dependent enzymes does not cause any inhibition of $O_2^-$ release, blockage of lipoxygenase (LOX) results in near elimination of the signal. This suggests that $O_2^-$ release is dependent on AA metabolism through the LOX pathway. However, confocal measurements of intracellular $O_2^-$ formation suggest that intracellular ROS are produced by a separate mechanism. 5) Tissue fluorometry techniques, using a fluorescence probe sensitive to hydrogen peroxide, showed that acute hypoxia also induces ROS in skeletal muscle.

These findings provide insight into understanding the potential role of ROS in physiologic and pathophysiologic responses to heat exposure and acute hypoxia in skeletal muscle.
Dedicated to my parents and my uncle
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VITA

Born – Beijing, China

1985-1988 ........................................ Beijing No.4 High School, Beijing, China

1992 ................................................ B.S., Peking University, Beijing, China

2000 ................................................ 1) Winner (First Place), the competition of poster research presentation on professional biological sciences, 2000 Edwards F. Hayes Graduate Research Forum;

                                  2) Winner (First Place), the oral research presentation, 2000 Biophysics Symposium at Ohio State;

                                  3) Winner of Travel Grant for research meetings, awarded by Biophysics Program at Ohio State

2002 ................................................ Winner of Student Research Award, WHOET (an international conference of Wound Healing---Oxygen & Emerging Therapeutics) 2002, USA;

                                  M.S., The Ohio State University, Columbus, Ohio
PUBLICATIONS

Research Publications


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CHAPTER 1

INTRODUCTION

1.1 Free radical biology

A free radical is defined as any species containing one or more unpaired electrons. The field of free radical biology has undergone three primary historical steps in the past 50 years (64). The first era started in 1954, when Commoner et al. discovered the presence of free radicals in a biological system (52). The second era started in 1969, when McCord and Fridovich discovered superoxide dismutase, and unveiled the important status of free radicals in biology (145). The third era began with extensive evidence showing the various effects of free radicals on nearly all biological systems, such as muscle relaxation, platelet adhesion, aging, cancer, cellular damage, and activation of the second messenger cGMP (91,92,149,178). Now at the beginning of the 21st century, the science of free radical biology has been growing rapidly and is having tremendous impacts on medicine, cell biology, physiology, anatomy, and pathology.

In the field of free radical biology, extensive studies have disclosed the important role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in biological systems. ROS generation occurs in viral infection, heat stress, hypoxia, as well as various diseases
such as atherosclerosis and obstructive sleep apnea (49,62,64,152,247). Most ROS are oxygen-centered or related radicals, such as superoxide (O$_2^-$) and hydroxyl radicals (\(\cdot\)OH), but interestingly, some ROS such as hydrogen peroxide (H$_2$O$_2$) are not free radicals, since H$_2$O$_2$ does not have unpaired electrons in the outer molecular orbit, the typical characteristic of free radicals. RNS are molecules derived directly or indirectly from nitric oxide (\(\cdot\)NO), an extremely important molecule in regulating most biological events (111,175,194,241,243). In the next section of this chapter, a general introduction to ROS and RNS will be addressed.

1.2 General introduction of ROS
Molecular O$_2$ is paramagnetic in the ground state, containing two unpaired but spin-paralleled electrons. This kind of structure would favor a univalent pathway of reduction, resulting in the formation of superoxide (one-electron reduction), hydrogen peroxide (two-electron reduction), hydroxyl radical (three-electron reduction), or water (four-electron reduction). The first three species are most common ROS, as described below.

1.2.1 Superoxide (O$_2^-$)
The O$_2^-$ molecule is a short-lived anion that is normally formed after one oxygen molecule accepts one electron from a reducing agent. O$_2^-$ is an anion molecule that is impermeable to membranes such as the mitochondria inner membrane. However, anion channels have been shown to be able to facilitate O$_2^-$ transport across the cell or mitochondrial membrane (139,230). O$_2^-$ is a conjugate base of weak acid and thus very
unstable in protonic solutions. $O_2^{-}$ tends to spontaneously dismute to hydrogen peroxide, particularly when pH is about 4.7 (17). $O_2^{-}$ can also act as both a reductant (\(E_o'\) for $O_2^{-}/O_2$ is about 0.33V) and an oxidant (\(E_o'\) for $H_2O_2/ O_2^{-}$ is about 0.87V) (70), thus expanding its reactions with many biological molecules, such as oxidations of both ascorbic acid and $\alpha$-tocopherol (159) and reductions of both ketones (76) and metal cations (171). The $O_2^{-}$-related equations (93) are listed below:

\[
\begin{align*}
O_2 + e^- & \rightarrow O_2^- \quad [1] \\
H_2O_2 & \rightarrow HO_2^* + e^- + H^+ \quad [2] \\
O_2^- + Fe^{3+} & \rightarrow Fe^{2+} + O_2 \quad [3] \\
H_2O_2 + Fe^{2+} & \rightarrow Fe^{3+} + OH^* + OH^- \quad [4] \\
O_2^- + H_2O_2 & \rightarrow OH^* + OH^- + O_2 \quad [5]
\end{align*}
\]

Equation [1] and [2] chemically describe the two possible mechanisms of $O_2^{-}$ formation, either by oxygen reduction or $H_2O_2$ oxidation. Equation [5], the Haber-Weiss reaction, is the net reaction of [3] and [4], illustrating the toxicity of $O_2^{-}$ by generating potent oxidant $OH^*$ in the presence of both $H_2O_2$ and iron (93). This is particularly important in the study of cellular damage, because the generation of $OH^*$ produced through equation
[5] can cause extensive protein and lipid oxidations. Furthermore, O$_2^-$ can attack iron-sulfur clusters, resulting in the release of ferrous iron, which can bind DNA before its reaction with H$_2$O$_2$. Thus, catalyzed by the iron bound in DNA molecule, the *OH produced by equation [4] would cause further damage and possible mutagenesis of DNA (93). Fortunately, in normal conditions, most iron molecules are not free in biological systems. These irons are stored in various sites such as ferritin and hemosiderin (104). However, in abnormal conditions such as acidosis or ischemia (137), these irons can be liberated from storage sites, leading to severe damage by facilitating free radical generation (104).

1.2.2 Hydrogen peroxide (H$_2$O$_2$)

H$_2$O$_2$ is diamagnetic since it has no unpaired electrons. Although it is not a free radical, it is one of the most important ROS. Compared to O$_2^-$, it is a stronger oxidant, and is much more permeable to cell or mitochondrial membranes by passive diffusion. The reactions related to H$_2$O$_2$ are listed below:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$ \[6\]

$$O_2^- + e^- + 2H^+ \rightarrow H_2O_2$$ \[7\]

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$ \[8\]
H$_2$O$_2$ can be generated in various ways, such as by the direct dismutation of O$_2$$\cdot$ from equation [6], by the univalent reduction of O$_2$$\cdot$ from equation [7], or by the divalent reduction of oxygen from equation [8].

H$_2$O$_2$ is one of the most stable ROS because it tends to react slowly with many bioorganic molecules. Compared to •OH (which will be discussed in the next section), H$_2$O$_2$ has much less reactivity. However, at high concentrations (i.e. mM level), H$_2$O$_2$ can cause severe damage on proteins, DNA, as well as lipid peroxidation (93). The major toxicity of H$_2$O$_2$ arises from the interaction between H$_2$O$_2$ and iron known as Fenton reaction, as shown in the next section.

1.2.3 Hydroxyl radical (•OH)

•OH is known as one of the most potent radicals and ROS. As a very strong oxidant, the attack of •OH to biological systems can cause extensive cellular oxidations (32). As mentioned in equation [5], •OH can be generated by the Haber-Weiss reaction. Another most important generation system for •OH is the Fenton reaction as described in the following equation:

$$\text{H}_2\text{O}_2 + \text{Fe}^{2+}/\text{Cu}^+ \rightarrow •\text{OH} + \text{OH}^- + \text{Fe}^{3+}/\text{Cu}^{2+}$$

Equation [9] illustrates reduced metals, such as iron or copper, have a tremendous tendency to react with H$_2$O$_2$, resulting in the formation of •OH. Similar to O$_2$$\cdot$, •OH is
also a short-lived molecule \((10^{-9} \text{ s in cells})\) and is impermeable to the membrane. However, it has a high reactivity and thus it can react with any molecules in its vicinity at diffusion-limited rates (the rate constant is as high as \(10^8 \text{ to } 10^{10} \text{ M}^{-1}\text{s}^{-1}\)) (93,123).

1.3 General introduction of RNS

RNS include nitric oxide (\(\cdot\text{NO}\)), peroxynitrite (\(\text{ONOO}^\cdot\)), nitrogen dioxide (\(\cdot\text{NO}_2\)), dinitrogen trioxide (\(\text{N}_2\text{O}_3\)), and alkyl peroxynitrates (LOONO). This section will focus on the two most important RNS: \(\cdot\text{NO}\) and \(\text{ONOO}^\cdot\).

For a long time, \(\cdot\text{NO}\) was believed to be a gas chemical with limited effects on biology. Its real biological role was not fully understood until the past 25 years, when \(\cdot\text{NO}\) was first characterized as an endothelial-derived relaxing factor (EDRF), released by endothelial cells in blood vessels to adjacent smooth muscle cells for relaxation. EDRF was later determined to be \(\cdot\text{NO}\), which has proved to be an important factor for regulation of blood pressure and platelet aggregation, for learning and memory as a neurotransmitter, as well as a multi-function biological messenger (98,151,210).

In an NADPH-dependent process, \(\cdot\text{NO}\) is generated during the oxidation of L-arginine to citrulline by nitric oxide synthase (NOS). There are three different isoforms of NOS, including endothelial cell NOS (ecNOS or NOS III), neuronal NOS (nNOS or NOS I), and inducible NOS (iNOS or NOS II). Both ecNOS and nNOS are \(\text{Ca}^{2+}\)-dependent and also called constitutive NOS (cNOS), while iNOS is normally induced by inflammation
and bacteria (98). Inhibition of NOS by N\textsuperscript{G}-substituted analogues of arginine such as L-NMA and L-NAME could result in reduced \textsuperscript{1}NO production associated with vasoconstriction, elevation of blood pressure, and reduction of blood flow (213).

As a free radical gas, \textsuperscript{1}NO is moderately stable in aqueous conditions. The \textsuperscript{1}NO molecule is small, uncharged and diatomic with one unpaired electron in the outer electron orbital. Therefore, \textsuperscript{1}NO has a great tendency to interact with other molecules with unpaired electrons, which are usually other free radicals such as O\textsubscript{2}\textsuperscript{•}. Similar to H\textsubscript{2}O\textsubscript{2}, \textsuperscript{1}NO can also diffuse freely across biological membranes (136).

Like many other biological molecules, \textsuperscript{1}NO is a double-edged sword. The beneficial effects from \textsuperscript{1}NO include: acting as a ubiquitous intercellular messenger and helping nonspecific host defense. The toxicity of \textsuperscript{1}NO is mainly from the diffusion-limited reaction of \textsuperscript{1}NO and O\textsubscript{2}\textsuperscript{•}, which results in the formation of another RNS named peroxynitrite (ONOO\textsuperscript{−}), a much more powerful and toxic oxidant. In other words, ONOO\textsuperscript{−} is a binary toxin that can be irreversibly generated almost spontaneously, whenever \textsuperscript{1}NO and O\textsubscript{2}\textsuperscript{•−} interact with each other (rate constant is as high as 6.7 x 10\textsuperscript{9} M\textsuperscript{−1}s\textsuperscript{−1}) (10). The reaction is listed below:

\[
\textsuperscript{1}\text{NO} + \text{O}_2\textsuperscript{•−} \rightarrow \text{ONOO}^\text{−}
\] [10]
In equation [10], the unpaired electron from $^\cdot\text{NO}$ rapidly interacts with the unpaired electron from $\text{O}_2^\cdot$. A large Gibbs energy (22 kcal/mol, which is basically equal to the energy released from the hydrolysis of two ATP molecules) is released when $\text{ONOO}^-$ is formed, thus explaining why this reaction is irreversible. Similar to $^\cdot\text{OH}$, $\text{ONOO}^-$ is not membrane-permeable. However, at physiological pH, 20% of $\text{ONOO}^-$ will be protonated in the form of peroxynitrous acid (ONOOH), and ONOOH is another strong oxidant that seems to be more permeable to cell membrane than $\text{ONOO}^-$ (10). ONOOH also facilitates the interferon-gamma-induced epithelial hyperpermeability (228). Behaving like $^\cdot\text{OH}$, the chemical attack of $\text{ONOO}^-$ / ONOOH on other molecules often reflects the bad side of $^\cdot\text{NO}$, since ONOO$^-$ / ONOOH can cause many cellular injuries by oxidizing iron/sulfur centers (resulting in the release of irons and further iron-mediated damage), protein thiols, and zinc fingers (10,94). ONOO$^-$ is not a free radical and can be very stable in alkaline solution or in solid state for a long time. Under acidic conditions, ONOO$^-$ can break down rapidly into $^\cdot\text{OH}$ and $^\cdot\text{NO}_2$, which are both potent oxidants. Therefore, the toxicity of ONOO$^-$ is tremendous. One of the most significant forms of damage by ONOO$^-$ is probably due to the tyrosine nitration in proteins, which is commonly found in lung biopsy specimens with sepsis, respiratory distress syndrome, pneumonia, and cardiac muscles with myocardial depression (10). This is because tyrosine nitration can lead to the deleterious protein dysfunction. For example, as negatively charged residues at ~ physiological pH, nitrotyrosine moieties in structural proteins such as cytoskeletal proteins, can cause serious mismatch in protein assembly, which is associated with many diseases (10).
1.4 Major downstream oxidations by ROS

ROS are able to cause oxidative damage to cellular constituents such as lipids, nucleic acids and proteins. The mechanisms of these forms of damage will be addressed in the following sections.

1.4.1 Lipid peroxidation

Cell membranes have tremendous amounts of polyunsaturated fatty-acids linked to phospholipids. These lipids are hydrophobic, supporting membrane structure and active membrane transport. Those unsaturated double bonds make their adjacent carbon-hydrogen bonds relatively weak. Thus, ROS including $O_2^-$, $^*OH$, perhydroxyl radical (HO$_2^*$), and conjugated peroxyl radical (LOO$^*$), can attack and oxidize these polyunsaturated lipids, resulting in loss of membrane integrity and function. For example, membrane fluidity, electrical resistance, membrane protein mobility, and the activity of ion pumps in the membrane, can all be greatly reduced due to the loss of polyunsaturated fatty acids by ROS attack (187). Particularly, $^*OH$ is one of the most active ROS in the initiation of lipid peroxidation. The chain reactions initiated by $^*OH$ are listed below:

$$\text{Lipid-H + } ^*\text{OH } \rightarrow \text{H}_2\text{O + Lipid}^*$$  \hspace{1cm} [11]$$

$$\text{Lipid}^* + O_2 \rightarrow \text{Lipid-OO}^*$$  \hspace{1cm} [12]$$
Lipid-OO$^\cdot$ + Lipid-H $\rightarrow$ Lipid-OOH + Lipid$^\cdot$ \[13\]

Lipid$^\cdot$ + Lipid$^\cdot$ $\rightarrow$ Lipid-Lipid \[14\]

Lipid-OO$^\cdot$ + Lipid-OO$^\cdot$ $\rightarrow$ Lipid-OO-Lipid + O$_2$ \[15\]

Lipid-OO$^\cdot$ + Lipid$^\cdot$ $\rightarrow$ Lipid-OO-Lipid \[16\]

Considering the whole process of lipid peroxidation, equation [11] is the initiation reaction, equations [12] and [13] are the propagation reactions, and equation [14]–[16] are the termination reactions. \textsuperscript{•}OH attack results in the abstraction of one hydrogen atom from the lipid and the formation of water and carbon-centered lipid radical, as shown in equation [11]. Then the active lipid radical takes up molecular oxygen to generate lipid peroxy radical (Lipid-OO$^\cdot$) as illustrated in equation [12]. This newly formed peroxy radical can attack another fatty acid molecule, resulting in initiation and propagation of another cycle of lipid peroxidation (equation [13]). Furthermore, in the presence of iron or copper, the lipid peroxides (Lipid-OOH), one product of equation [13], can decompose into either peroxy (lipid-OO$^\cdot$) or alkoxy (lipid-O$^\cdot$) radicals (123). Normally, lipid peroxidation is measured by its own by-products such as malondialdehyde (MDA), 4-hydroxyalkenals (4-HNE), thiobarbituric acid reactive substances (TBARS), and lipid hydroperoxides (LH) (123,187).
1.4.2 DNA damage by ROS

This topic is mostly studied with radiation technology. Endogenous ROS can attack DNA to generate lesions by modifying bases such as by hydrogen abstraction. These modified bases can potentially damage the integrity of the genes. For example, 8-hydroxyguanine (8-OH-Gua) is one of the most common lesions caused by ROS attack. The formation of 8-OH-Gua may lead to erroneous GC→TA transversions during DNA replication, resulting in possible mutagenesis, atherosclerosis and carcinogenesis (165). Other modified bases by ROS include 5-hydroxyuracil (5-OH-Ura), 5-hydroxycytosine (5-OH-Cyt), and 8-hydroxyadenine (8-OH-Ade), which all have premutagenic function (234).

1.4.3 Protein oxidation by ROS

A range of studies have demonstrated that protein damage as occurs with disulfide cross-linking may be a direct result of ROS attack and may have profound influences on enzyme activity, the conformation, hydrophobicity and degradation of protein, cell function, and cytolysis (46,57,237). Oxidative modifications of proteins by ROS make these proteins more susceptible to proteolytic reaction. These modifications include oxidation of protein sulfhydroyl moieties, site-specific oxidation of arginine, lysine and histidine, and oxidation of methionyl residues (46,215,237). In some conditions such as ischemia-reperfusion and inflammation, these modified proteins may accumulate to a significant level, and result in severe cell dysfunction. If the amino acid modified by ROS is the key residue for the enzymatic function, the influence of such a minor modification will be tremendous. Fortunately, in normal cells, most modifications of
amino acid residues are non-specific, leading to much less cellular damage. Furthermore, oxidatively damaged proteins generally tend to be broken down rapidly by proteases. There is also much less possibility of chain reactions in protein oxidation compared to lipid peroxidation. However, the lipid radicals induced by ROS are able to cause protein damage (46,57,215,237). It is worthy noting that some enzymes with antioxidant activities, such as glutathione peroxidase and catalase, can be inactivated by ROS under proper conditions, resulting in possible imbalance between ROS and antioxidant defense system (75). Some critical enzymes in the citric cycle, such as mitochondrial aconitase, can also be inactivated by ROS as tested for both in vivo and in vitro experiments (11), suggesting a possibility that mitochondrial function may be dampened by its own production of ROS.

1.5 ROS and \textsuperscript{3}NO act as signaling molecules

Although ROS are frequently associated with tissue damage and cytotoxicity, accumulating evidence has demonstrated that low levels of ROS are required for basic cellular function and signaling. Being important biological messengers and modulators, ROS can affect growth promotion or inhibition, necrosis, apoptosis, induction or suppression of many genes, and activation of protease activities. Specifically, ROS can influence the activation of mitogen-activated protein kinases (MAPK), regulation of both nuclear factor \textsuperscript{kB} (NF\textsuperscript{kB}) and activator protein-1 (AP-1), activities of Jun-N-terminal kinases (JNK), and formation of inflammatory mediators and adhesion molecules (39,59,102,201). Thus, the inhibition of ROS can significantly attenuate these effects in
signaling cascades such as the activation of JNK and AP-1, as well as in gene transcription (168,201).

Protein phosphorylation, one of the basic mediators of cell signaling, can also be induced by ROS. For example, the level of tyrosine phosphorylation can be markedly increased after ROS stimulation in Jurkat cells (198). This is because two members of the Src family, p56\textsuperscript{lk} and p59\textsuperscript{fyn}, can be activated by ROS. Furthermore, the activities of p58c-fgr and p53/56lyn tyrosine kinases can also be elevated by ROS (198). Therefore, protein phosphorylation, a fundamental process of cell signaling cascades, is highly redox-sensitive and subject to modifications by both ROS and antioxidants.

\chem{\cdotNO} also has tremendous influences on signaling cascades involved in the regulation of force generated by vascular smooth muscle. These \chem{\cdotNO}-mediated mechanisms include the inhibition of mitochondrial respiration, the activation of cGMP formation by soluble guanylate cyclase (sGC), and the modulation of vasoactive mediator release. However, ROS can also modulate force generation by stimulating the production of vasoactive prostaglandins and activating sGC as well as protein kinase C (PKC). The stimulation of sGC by \chem{\cdotNO} can be reduced by \chem{O_2\cdot} because of the formation of ONOO\textsuperscript{−}.

\chem{\cdotNO} can inactivate catalase, resulting in more ROS (H\textsubscript{2}O\textsubscript{2}) production (238). However, \chem{\cdotNO} may inactivate enzymes that are able to produce ROS, particularly NADPH oxidase, one of the major ROS generators, thus leading to the possible attenuation of intracellular
oxidant levels (77). Therefore, the interaction among ROS, \( \cdot \text{NO} \) and antioxidant systems tend to make this biological process much more complicated.

1.6 Biological sources of ROS

A number of potential cellular sources for ROS have been proposed in skeletal muscle. One likely source of elevated ROS production in various models is the mitochondrion. Mitochondria are believed to produce basal levels of ROS by the electron leakage from electron transport to oxygen during normal metabolism (44). During respiration, ubiquinone (CoQ) accepts electrons from both complex I and II via two sequential one-electron reductions to ubisemiquinone and ubiquinol, defined as the Q cycle. Then the reducing equivalents pass through complex III, cyt c and complex IV. In all, four electrons from the electron transport are eventually transferred to molecular oxygen to form water. During the whole process, there is about 2% of the electron flow that may leak out from possible sites such as Q cycle to reduce oxygen to \( \text{O}_2^{\cdot -} \) (22). When cells or tissues are under stressful or diseased conditions, the electron leakage may be greatly increased, thus causing more \( \text{O}_2^{\cdot -} \) formation (11,44). Unfortunately, mechanisms associated with changes in mitochondrial function and metabolism regarding ROS generation are not fully understood, but they might be consistent with those conditions described by Nohl, et al. (161).

Other sources are also possible. For example, the diaphragm that we used as a skeletal muscle model has a large distribution of capillary endothelium, which is at least twice that of comparable muscle fiber population (108). Endothelial cells are known to be
potential sources of ROS generation through membrane-bound NADH and/or NADPH oxidases (56,153). ROS produced in endothelium may mediate vascular responses to shear stress and other stimuli, which may be important in local vascular regulation (56).

Nethery et al. have shown that phospholipase A2 activation seems to be a necessary step in the increase of ROS production during skeletal muscle stimulation (157). The activation of phospholipase A2 may result in ROS formation by generating arachidonic acid from available membrane phospholipids. Arachidonic acid may interfere directly with electron transport (51) or produce ROS via cyclooxygenase pathways (164,236).

In the downstream of arachidonic acid metabolism, there are also two other major pathways besides cyclooxygenase: one is cytochrome P450-dependent monooxygenase; the other is lipoxygenase (LOX). Cytochrome P450 enzymes are known as ROS generators (20,170), which may either directly reduce oxygen to $\text{O}_2^{-}$ or mediate the electrons transferring from substrate to oxygen (11). LOX has been shown to be capable of directly contributing to ROS formation (124,127,138,239,240), but the mechanisms are not fully understood. There are at least two possible processes. First, non-heme iron in LOX can cycle between ferrous (Fe$^{2+}$, inactive) and ferric (Fe$^{3+}$, active) forms during the catalysis of AA to leukotrienes, resulting in the formation of hydroperoxide radical intermediates by the addition of oxygen molecules to AA substrates. During this process, it is possible that Fe$^{2+}$ could directly transfer one electron to molecular oxygen to form $\text{O}_2^{-}$ (24). Second, the products of LOX activities such as leukotrienes may play an
autocrine or paracrine role in stimulating ROS formation by secondary mechanisms (239,240).

Furthermore, xanthine oxidase (56) and nitric oxide synthase (NOS) (173) have also been presented as potential sources of ROS in skeletal muscle and other tissues. It is possible that the activity of these enzymes and/or the influx of intracellular Ca\(^{2+}\) may be uniquely affected during oxidative stress; however, their role has not been fully identified yet, particularly in muscle. Other organelles that can produce ROS include peroxisomes, endoplasmic reticulum, plasma membrane and nuclear membrane. Furthermore, polymorphonuclear cells and macrophages resident in the tissue can also be ROS generators (61).

### 1.7 Antioxidant defense mechanisms

The cell has developed an elaborate antioxidant system, including various enzymes or molecules to scavenge intra- and extracellular ROS produced either in normal conditions or oxidative stress. These include superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, the thioredoxin system, and nonenzymatic antioxidants or scavengers. However, it is highly likely that the endogenous antioxidant system may be overwhelmed under conditions such as oxidative stress, leading to more ROS-induced cellular damage.

#### 1.7.1 Superoxide dismutase (SOD)

SOD was first discovered in 1969 by McCord and Fridovich (145), shedding insight into the endogenous antioxidant system. SODs are metalloenzymes that catalyze the
dismutation of O$_2$\textsuperscript{−} to H$_2$O$_2$ at diffusion limited rates ($\sim$ 2 x 10$^9$ M$^{-1}$s$^{-1}$). In this study (see chapter 2), we applied SOD to our bath solutions to scavenge O$_2$\textsuperscript{−}. This reaction is described below:

$$2\text{O}_2\textsuperscript{−} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$  \[17\]

SODs can be found in all oxygen-consuming organisms and are normally divided into three categories based on the metal located at the active site. The copper-zinc isoforms (Cu,ZnSODs) are normally present in the cytosol of eukaryotic cells; the manganese isoforms (MnSODs) are found in prokaryotes and the mitochondrial matrix of eukaryotes. The iron-containing isoforms (FeSODs) are mainly distributed in prokaryotic cells (11,217). However, by the analysis of the amino acid sequence data, these three SOD isoforms can be grouped into two major families, the Cu,ZnSOD and the Fe/MnSOD (93). There is also the existence of extracellular Cu,ZnSOD, a secretory glycoprotein located in plasma and tissues (11,217). As a protein of 32-150 kD, SOD dimers or tetramers cannot pass the cellular membrane freely. Note that the reaction between $^*$NO and O$_2$\textsuperscript{−} is also in the diffusion-limited rate. Therefore, theoretically, there is going to be a competition for O$_2$\textsuperscript{−} between SOD and $^*$NO, depending on various conditions. More interestingly, lower concentrations of SOD have a protective effect on cells by ameliorating ROS-induced injuries. However, if the SOD concentration is too high, sometimes this may not benefit tissue possibly due to the “overscavenging effect.”
For example, termination of lipid peroxidation may be inhibited by a high dose of SOD (122,166).

1.7.2 *Glutathione peroxidase (GPX)*

The selenium (Se)-dependent GPX is an 85kD tetramer, whose function is to convert H₂O₂ to water using the hydrogen atoms from reduced glutathiones (GSHs) as shown in the following equation:

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \quad [18]
\]

GPX is very selective to GSH utilization, but much less specific to peroxide substrates, which include H₂O₂, various organic peroxides and some lipid peroxides. These peroxides can also be good substrates for GPX. Thus the biological antioxidant effect from GPX is somewhat extensive. Furthermore, an artificial GPX mimic named ebselen has been used in some studies and will be used in this one to scavenge H₂O₂ (see chapter 5). GSSG, the product of this antioxidant reaction, can be used to regenerate fresh GSH through GSH reductase (a 105 kD dimer) in the presence of NADPH, which is shown as:

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \quad [19]
\]

The NADPH required in this equation can be regenerated by glucose-6-phosphate dehydrogenase in the hexose monophosphate shunt. GSH can also be synthesized
through the $\gamma$-glutamyl cycle by GSH synthetase. The ratio of GSH/GSSG is normally used as an important biological index of cellular redox status (122,235).

When cellular Se is low, the non-Se-containing GPX named GSH-S-transferase (GSTs), will take the place of GPX, and act on the cellular hydroperoxides. However, interestingly, GST cannot reduce H$_2$O$_2$ (129).

### 1.7.3 Catalase

Catalase is a 24 kD homotetrameric antioxidant enzyme with a heme-iron active center. Catalase is mainly located in peroxisomes, one of the major sites for intracellular H$_2$O$_2$ formation. Catalase can also be found in mitochondria from cardiac muscles (235). The main function of this enzyme is to decompose toxic H$_2$O$_2$ into water and oxygen as shown in the following equation:

$$2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \quad [20]$$

Compared to GPX, catalase tends to reduce small peroxides such as H$_2$O$_2$, but has no effect on larger molecules such as lipid hydroperoxides. Catalase was used in this study to scavenge extracellular H$_2$O$_2$ (see chapter 2). Catalase is a low-affinity but high-capacity enzyme, perfectly suited for H$_2$O$_2$ scavenging at localized organelles such as mitochondria and peroxisomes where higher level of ROS can be produced. However, GPX is a high-affinity but low capacity antioxidant enzyme well distributed in cells, and can work on peroxides with large molecular weights (235).
1.7.4 Thioredoxin system

Thioredoxin (TRX) is a low molecular weight protein (11.5 kD) located in cytosol, nucleus and mitochondria in both eukaryotes and prokaryotes. The cystein residues at conserved Trp-Cys-Gly-Pro-Cys-Lys sequence in TRX can undergo reversible reduction and oxidation catalyzed by NADPH-dependent TRX reductase. TRX is used as a hydrogen donor for many enzymes such as TRX peroxidase, tyrosine phosphatase, sulfoxide methionine reductase, and ribonucleotide reductase. TRX also has a stimulatory effect on growth and an inhibitory effect on apoptosis (11,163,198).

The TRX system is primarily composed of TRX reductase and TRX peroxidase. The major function of TRX reductase is to catalyze the reduction from oxidized TRX back to reduced TRX with the electrons donated from NADPH. Reduced TRX can be used to convert H$_2$O$_2$ to water with the catalysis of TRX peroxidase. The TRX system plays a critical role in the protein thiol redox control by reducing oxidized thiols. As an electron donor, TRX is extensively involved in the regeneration of many antioxidants such as vitamin C, lipoic acid, ubiquinone, and selenium-containing substrates (11,163,198).

Those mentioned above are the most common antioxidant enzymes in the field of free radical biology. There are also many other antioxidant enzymes including myeloperoxidases, eosinophil peroxidases, lactoperoxidases, ovoperoxidases, and thyroid peroxidases, which work together to form a strong antioxidant defense network.
1.7.5 Nonenzymatic antioxidants or scavengers

The non-enzymatic antioxidants or scavengers work in concert with those antioxidant enzymes listed above, to provide the maximal protection against oxidative injuries. The chemical properties of any antioxidant determine its solubility, and thus its biological localization. Lipid soluble antioxidants such as vitamin E are primarily localized in the hydrophobic part of the membrane to prevent possible damage caused by ROS-induced lipid peroxidation. Water-soluble antioxidants such as vitamin C and uric acid, are mainly localized in cytosol or extracellular fluid, acting on ROS that are generated from the water phase (199). The most common nonenzymatic antioxidants or scavengers are summarized below.

1.7.5.1 Vitamin E

The category of vitamin E antioxidants includes all the tocol and tocotrienol derivatives with the activities of α-tocopherol. Vitamin E is rich in vegetable oils, unprocessed cereal grains and animal meat. The major role of vitamin E is to protect polyunsaturated lipids within the membrane or plasma lipoproteins from ROS attack, thus preventing propagation of oxidative damage. The mechanism of the chain-breaking antioxidant vitamin E is shown below:

$$\alpha-T + RO_2^* \rightarrow RO_2H + \alpha T^*$$  \[21\]
\( \alpha \)-T represents vitamin E (\( \alpha \)-tocopherol). \( \text{RO}_2^* \) represents lipid peroxy radicals. In this reaction, the phenol group of vitamin E converts \( \text{RO}_2^* \) to lipid hydroperoxide (\( \text{RO}_2^\text{H} \)), a product with much less toxicity when compared to its radical form, \( \text{RO}_2^* \) (199).

1.7.5.2 Vitamin C

Also named ascorbate, vitamin C is highly water-soluble, and can be synthesized from glucose precursors, though this does not occur in humans. Ascorbate has a strong reducing capacity and can donate electrons to many oxidized irons such as \( \text{Fe}^{3+} \) and \( \text{Cu}^{2+} \), resulting in the generation of transition metal ions involved in biological redox cyclings. Ascorbate can also interact with the phenol group of vitamin E located at the water-membrane interface, facilitating the activities of vitamin E. The oxidation product of ascorbate can be reduced by GSH, suggesting a direct interaction between ascorbate and other antioxidant networks (199).

1.7.5.3 \( \alpha \)-lipoic acid

\( \alpha \)-lipoic acid is present in anionic form at physiological pH. \( \alpha \)-lipoic acid can be readily absorbed by the cells where it is reduced to its dithiol form, named dihydrolipoate (DHLA). Since the redox potential of \( \alpha \)-lipoic acid-DHLA is \(-320\) mV, DHLA is a strong reductant. For example, it can reduce GSSG to GSH. Extracellular DHLA can also donate electrons to cystine residues at the outer layer of the cell membrane, resulting in the formation of more reduced form, named cysteine. Furthermore, \( \alpha \)-lipoic acid can facilitate glucose metabolism by stimulating glucose uptake. \( \alpha \)-lipoic acid also plays an
important role in mitochondrial oxidative metabolism by being present in a bound lipoyllysine form in mitochondrial proteins, including α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (199).

1.7.5.4 Carotenoids

Carotenoids are 40-carbon long chain molecules with multiple double-bonds. These include lycopene, lutein, astaxantin, violaxanthin, zeaxanthine, α-, β-, γ-carotene, and β-carotene-5,6-epoxide. The major function of carotenoids is to scavenge ROS and protect cells from oxidative damage (199).

1.7.5.5 Ubiquinone

Ubiquinone is also called coenzyme Q₁₀, which is well known as a critical component of mitochondrial electron transport. Ubiquinone is widely distributed in plasma membranes, intracellular membranes and low-density lipoproteins. The major antioxidant activities of ubiquinone are due to its involvement in preventing lipid peroxidation and regenerating vitamin E (199).

1.7.5.6 Other nonenzymatic antioxidants and scavengers

Se is most well known, probably due to its critical role in the biological activities of GPX. However, Se can also exert direct antioxidant effects on other biological systems (199). N-acetyl-L-cysteine (NAC) is not an endogenous antioxidant but often used in studies of free radical biology (60,199). This is because NAC has a capacity to maintain the intracellular thiol pool and prevent GSH oxidation. Uric acid is also a powerful
antioxidant, acting on singlet oxygen, \( \cdot \text{OH} \), lipid radicals, as well as binding transition metals (95,199). Other biological molecules include glucose which can scavenge \( \cdot \text{OH} \), cysteine which tends to donate electrons from its thiol moiety, pyruvate which is a scavenger of \( \text{H}_2\text{O}_2 \), histidine which can scavenge both singlet oxygen and \( \cdot \text{OH} \), and albumin which prevents the Fenton reaction by binding transition metals (95,199).

A number of chemicals can work as ROS scavengers. Dimethylthiourea (DMTU) is a powerful scavenger of \( \cdot \text{OH} \), \( \text{H}_2\text{O}_2 \) and \( \text{HOCl} \). Mannitol can react with \( \cdot \text{OH} \) to form a nonreactive mannitol dimer. Some scavengers act on ROS by an indirect way. For example, deferoxamine can impede ROS formation from the Harber-Weiss reaction by chelating free iron. N-2-mercaptopropionylglycine (MCPG), can function as both a sulfhydryl donor for GSH and a scavenger for \( \text{O}_2^{*-} \) and \( \cdot \text{OH} \). Thus, MCPG is able to exert maximal protection for cells from oxidative damage (122).

1.8 Biological effects of ROS and \( \cdot \text{NO} \) in skeletal muscle

Both ROS and RNS are important biological molecules, but their role on skeletal muscle has not been fully understood. Therefore, in the following sections, the interactions among ROS, RNS and the function of skeletal muscle as well as corresponding mechanisms will be addressed.

1.8.1 ROS and skeletal muscle

Reid and associates have made thorough studies on ROS produced in skeletal muscle
They concluded that ROS can be produced both intra- and extracellularly in both resting and contracting skeletal muscle. They also found that low levels of ROS play a critical role for both low-frequency fatigued and unfatigued muscle (181-183). However, higher levels of ROS may contribute to the force reduction during intensive fatiguing exercise (150,247). Furthermore, a wide variety of antioxidants such as catalase, SOD and dithiothreitol, can markedly attenuate the process of fatigue (181,182,205).

Other ROS-related stress models of skeletal muscle have been studied. Mohanraj et al. have shown that antioxidants including dimethyl sulfoxide, NAC, SOD and Tiron, all have a protective role for skeletal muscle during hypoxia (150). This suggested, indirectly, that significant levels of ROS must be produced during hypoxia. Thus, research for a direct evidence of hypoxia-induced ROS in skeletal muscle is imperative. In chapter 5 of this dissertation, I will describe fluorescence techniques that are used for the first time to show the intracellular ROS generation in skeletal muscle during hypoxia, which is highly consistent with previous results in other tissues (55,231). Another ROS-related stress model of skeletal muscle is heat stress (247). In chapter 2, I will demonstrate both intra- and extracellular production of ROS in skeletal muscle during heat stress. I will also show that antioxidants such as Tiron and SOD can significantly attenuate ROS production in heat stress. Therefore, the fact that ROS are generated in conditions of rest, fatigue, hypoxia and heat stress strongly suggests the tight linkage between skeletal muscle activities and mechanisms of ROS generation.
1.8.2 *NO and skeletal muscle

*NO has been shown to have a significant effect on the function of skeletal muscle. *NO lowers the myofibrillar Ca\(^{2+}\) sensitivities by impairing Ca\(^{2+}\) activation of actin filaments (6). NOS (nNOS and ecNOS) was found to be located in the sarcoplasmic reticulum (SR) membrane, possibly playing an inhibitory role in SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release in skeletal muscle (81,148). The negative inotropic effect of *NO on diaphragmatic contractile dysfunction has been illustrated by the fact that *NO blockers which suppress the large amounts of *NO produced by iNOS during sepsis, prevent muscle failure by reducing *NO release (194). It is worth noting that these experiments were done almost exclusively in ambient oxygen tension (PO\(_2\) > 150 mm Hg) \textit{in vitro}. However, intracellular PO\(_2\) is \sim 10 mm Hg in skeletal muscle (66). Under this PO\(_2\) condition \textit{in vivo}, Eu et al. found that *NO at submicromolar levels, can activate ryanodine receptors (RyRs) to release Ca\(^{2+}\) by S-nitrosylation of a single cysteine residue in RyR1 in rabbit skeletal muscle (66). Aghdasi et al. found that in skeletal muscle, low levels of *NO impede the oxidation of the Ca\(^{2+}\) release channel, whereas high levels of *NO oxidize it (1). Therefore, cautious steps should be taken when considering interactions between *NO concentrations and PO\(_2\) during these kinds of experiments.

Other functional and biochemical effects of *NO on skeletal muscle include limitation of oxygen consumption (109), reduction of energy supply by *NO inhibition on mitochondrial cytochrome c oxidase (50), and stimulation of the glucose transport during exercise (7).
1.8.3 Interaction between ROS and *NO in muscle function

Both ROS and *NO are common biological mediators, which can be either produced intracellularly or released extracellularly by skeletal muscle (180-182,247). Both of them can directly modulate cellular processes by modifying redox-sensitive proteins. Therefore, the balance between activation and inactivation of the activities of both ROS and *NO supports a “dynamic redox homeostasis.” One special situation should be noticed with regard to the potential interaction between ROS and *NO. In ambient oxygen tension, the increase of ROS in stressed muscles may significantly interfere with muscle depression induced by *NO. This could happen in three ways: 1) ROS can facilitate SR Ca\(^{2+}\) release by oxidation of thiol moieties (33); 2) \(O_2^-\) can react with *NO to form ONOO\(^-\), which is a strong oxidant causing the S-nitrosylation on thiol sites in the SR and possibly at other locations, thus promoting Ca\(^{2+}\) release and muscle contraction (111); 3) *NO tends to suppress muscle contraction (111). Thus, these effects make the study of muscle function complicated. More interestingly, NOS may produce both superoxide and *NO under certain conditions. For example, when NOS is substrate-deficient for L-arginine, there is a greatly increased probability of peroxynitrite generation at the reactive site, in part, because of the proximity of these two reactants (241).

1.9 Exploration of the role of ROS in cellular stress

The role of ROS in biology is particularly complex because not only can they initiate oxidation and damage to a variety of cellular components, but they also can appear to
play important roles in mediating cell signaling events in response to stress. For example, ROS activate a number of transcription factors such as nF-κB (200), upregulate stress proteins such as HSP\textsubscript{70} (209), and cause increased expression of antioxidants such as manganese-SOD (242). ROS can also induce acute changes in glucose uptake and metabolism (19) and alter mitochondrial function, possibly playing a role in cellular O\textsubscript{2} sensation (45).

Particularly, there are at least three common stresses associated with exercising muscles, including fatigue, heat stress, and hypoxia. Fatigue (defined as the inability to maintain muscle contractile function at a constant workload)-induced ROS has already been studied extensively (117,182). However, there is lack of direct evidence of heat- or hypoxia-induced ROS generation. I believed these two stresses are also very important in muscle physiology. During exercise, the muscle is a natural heat generator, resulting in a tremendous temperature elevation in local exercising muscle (27,28). At the same time, due to a large oxygen demand, the intracellular oxygen level becomes very low, resulting in hypoxic cellular conditions (186). Therefore, the possibility of ROS formation could be high during these processes and will be discussed in section 1.9.1 and 1.9.2.

To study the mechanisms of these two major cellular stresses, heat stress and hypoxia, an intact model system was used in this dissertation research, in isolated rodent diaphragm. There are a number of reasons that this model is ideal for studying ROS formation in stressful environments. First, it is not an isolated cell but an intact tissue with a normal phenotype that is studied shortly after removing it from a healthy animal. Most studies of
ROS formation are in isolated cell lines, which cannot be considered normal. Second, it is an extremely hardy tissue that sees stressful stimuli that would damage less robust cells under conditions of exercise. Third, because of its geometry, it is possible to study it with advanced optical technology such as tissue fluorometry and laser scan confocal microscopy, in a microenvironment that can be reasonably well controlled. Fourth, diaphragms have been shown to generate considerable ROS under a variety of conditions (181,182).

1.9.1 Heat stress

Heat stress is chosen as the first stress model in this research in part because temperature is an extremely important factor affecting biological systems. Heat-stress related death, normally associated with heatstroke, has been of recent concern, particularly among young athletes. A number of athletes have died of heat-related syndromes over the past decades. The most famous example is Korey Stringer, a professional football player who died from heatstroke at the end of July, 2001. Considerable research has been done on temperature effects on muscles. In the 1970’s, some researchers studied the tissue temperature and oxygen consumption after exercise and found O2 consumption increases to a high level right after exercise. This may be related in part to the increased temperature of the muscle, as well as mechanisms of O2 debt due to lactate accumulation (28). At higher body temperature, skeletal muscles exhibit a lower level of phosphorylative efficiency and a higher respiratory rate (26,27). Furthermore, higher temperatures have significant effects on mitochondrial function. From 25°C to 45°C, as the mitochondrial oxygen consumption increases ~ 2.5 fold, the inhibition of respiration
rate by oligomycin also increases ~2 fold and the oligomycin-sensitive mitochondrial ATPase activity increases ~ 4 fold (27). Many researchers have focused their interest on a narrow but crucial temperature range between 41°C and 43°C, most often at 42°C, which is known as a threshold heat-stress temperature, because myocytes and most other cells can be significantly affected at this very range. For example, heat stress has large influences on energy metabolism of exercising muscle, causing an increase of ATP utilization and phosphocreatine (CrP) depletion (69). Heat stress can change the contractile machinery of skeletal muscles through inactivating the troponin-tropomyosin regulation system (179). Hall et al. have demonstrated that whole body heat stress causes a significant and potent elevation in ROS and *NO formation due to the apparent increased activities of xanthine oxidase and NOS (89). Furthermore, one important consequence of heat stress is the induction of a set of polypeptides named heat shock proteins (HSP), which protect cells from damage due to stress (97,134). Elevated temperature may also cause cells to undergo severe oxidative stress, resulting in over-production of HSP, since the progressive uncoupling of mitochondria, the increasing ubisemiquinone concentration, and the elevation of HSP70 mRNA could be observed after intensive exercise (193). Previous research has shown that ROS could be related to the induction of HSP in heat stress (83). In skeletal muscle, the damage to protein from ROS induced by heat stress may be alleviated by HSP that may have been originally induced by ROS (65). Consequently, studying the dual role of ROS makes study of heat stress in skeletal muscle interesting and worthwhile. Overall, heat stress, HSP and ROS are closely related. However, many questions remain to be answered. For example, regarding heat stress, there has never been any direct evidence of ROS generation or the
molecular mechanisms of ROS formation. Research on these problems will be a primary focus of this dissertation.

1.9.2 Hypoxia

Another stress model addressed in this dissertation is hypoxia. Hypoxia has many definitions but can be defined in this setting as a deficiency of cellular oxygen delivery for a given O₂ demand. Although hypoxia is common in conditions of exercise, if prolonged or intense, it can produce tissue damage. Hypoxia/reoxygenation is commonly thought to produce ROS during reoxygenation. Whether ROS is generated during hypoxia is controversial. This is because many people believe that the cellular oxygen, the source of most forms of ROS, may be greatly decreased during hypoxia. Thus, they tend to favor the idea that ROS generation is more likely to occur during reoxygenation, since at this time point there is not only a significant accumulation of reducing compounds such as NADH but also a sufficient level of O₂ that can be reduced to O₂⁻. However, Vanden Hoek et al. (231) and Damerau et al. (55) have observed increased ROS production during hypoxia prior to reoxygenation in heart studies. This is a good example of one of the many paradoxes of free radical biology in which too much or too little O₂ can both cause ROS formation (49). In our laboratory, previous studies have shown that antioxidants protect rat skeletal muscle function under hypoxic conditions, suggesting the possibility that ROS generation during hypoxia plays a role in muscle contractile function (150). The enzymatic activities of xanthine oxidase, a ROS generator, can be elevated by excess Ca²⁺ influx induced by hypoxia (25), suggesting the involvement of ROS in hypoxia. Furthermore, during exercise, myocytes are situated in
more or less hypoxic conditions due to a large amount of O₂ demand, which is a common phenomenon in exercised skeletal muscle (186). Therefore, it is imperative to find any direct evidence of ROS induced by hypoxia in skeletal muscle, which will be discussed in chapter 5 of this dissertation.

1.9.3 Methods for detection of ROS

Currently, intracellular ROS can be detected by several methods, including 1) spin trapping techniques such as electron spin resonance (ESR) spin trapping, NMR spin trapping, and MRI spin trapping; 2) measurements of products of lipid peroxidation, DNA damage, and protein oxidation by ROS attack; 3) chemiluminescence; 4) fluorescence. Extracellular ROS release can be detected by cytochrome c or fluorescent probes.

1.9.3.1 Spin trapping techniques

Spin traps usually have no paramagnetic properties. However, when these traps react with ROS or *NO, they become relatively stable spin adducts which are paramagnetic. The advantage of spin traps is that free radicals can be trapped into relatively stable paramagnetic molecules with unique electron spin resonance that can be readily assigned to a specific radical species with a high sensitivity (15). In other words, spin traps are designed for rapid reaction with short-lived free radicals to form the longer-lived radical-adducts in order to build up detectable concentrations. The spin trapping technique has been widely used in the study of the oxidative burst of neutrophils (103,221), septic shock-induced *NO formation (78), and thiol or free radical levels in ischemia/reperfused
hearts (162). The primary limitation of the use of spin traps is the lack of sufficient stability of the adducts. Another limitation of spin traps is the necessity of using a high concentration (range from 10-50 mM) for an effective trapping of ROS. In such a high dosing, the toxicity and antioxidant or prooxidant effects of the trap cannot be ignored. Furthermore, if the spin traps are used in biological systems, the intracellular components may react with the spin adduct, resulting in a further decrease of adduct stability. For example, 5-(diethylphosphoryl)-5-methyl-1-pyrroline N-oxide (DEMPO) is often used as a spin trap for \( \cdot \)OH but the DEMPO/\( \cdot \)OH adduct is very unstable in whole blood compared to buffer, because the blood cells reduce the paramagnetic adduct to ESR insensitive products, usually the corresponding hydroxylamine (15).

1.9.3.2 Measurements of oxidative damage associated with lipid peroxidation, DNA oxidation, and protein oxidation

Many ROS are shot-lived, thus making the direct measurement of ROS difficult in biological systems. However, ROS formation can still be traced by measuring the damage products by ROS attack. In the process of lipid peroxidation induced by ROS (see section 1.4.1), the major breakdown product, named malondialdehyde (MDA), can be measured by thiobarbituric acid (TBA) assay. TBA reacts actively with MDA to form TBA-MDA conjugation product, which can be easily measured spectrophotometrically (90). However, the TBA assay has some major limitations: 1) the absorbance peak of TBA-MDA is at 532 nm, which overlaps with the absorbance peak of many endogenous pigments, thus often resulting in overestimation; 2) TBA is not only sensitive to MDA,
but also sensitive to sugars, amino acids, and many aldehydes, thus potentially leading to erroneous data analysis (90).

Another ROS-related damage is DNA oxidation, which has been discussed in section 1.4.2. Oxidized bases such as 8-OH-Gua, 5-OH-Ura, and 5-OH-Cyt can be measured by high performance liquid chromatography (HPLC) or gas chromatography-mass spectroscopy (GC-MS) (90). However, some artifacts can be generated during the measurements. For example, GC-MS methods require DNA to be derivatized, and this can result in the artificial oxidation of DNA (90).

A third ROS-induced damage is protein oxidation, which has been discussed in section 1.4.3. The most common parameters used to monitor protein damage by ROS attack include oxidized amino acid residues, protein carbonyls, S-thiolation, and thiol/disulfide redox status. The oxidized products of amino acids can be detected by HPLC or GC-MS after proteins are broken down into individual residues (90). ROS attack can also cause the formation of protein carbonyl moieties (protein–C=O). The reactant, 2,4-dinitrophenylhydrazine, can combine with carbonyl groups to form a detectable complex (absorbance peak at 370 nm), thus it is widely used as a common assay for the spectrophotometric detection of protein carbonyl groups (90). S-thiolation, defined as a ROS-mediated linkage between GSH and the target protein, can severely modify the protein structure and its corresponding function (90). In order to detect S-thiolation, the radiolabeled cystine is loaded into the cultured cells, where it is transported into the intracellular compartment, reduced to cysteine, and then incorporated into radiolabeled
GSH that is used for the synthesis of S-thiolation (90). Thus, monitoring the radiolabeled S-thiolation can be an indirect way to assay the intracellular ROS formation. The thiol/disulfide redox status can be detected either by radiolabeled probes or ESR methods (90). However, these methods lack specificity and reproducibility. For example, even in normal conditions, there is a significant level of oxidized amino acids and protein carbonyl formations, thus increasing the difficulty to differentiate these products. The measurement of S-thiolation is far too indirect, which could possibly involve many unknown variables during cell culture or sample preparation (90). Furthermore, in living tissues, the oxidized proteins are likely to be decomposed rapidly by various proteases rather than to accumulate to a detectable level, thus resulting in a greatly decreased sensitivity (46).

Overall, although useful in many studies, these methods are all indirect measurements of ROS formation, with less specificity and accuracy as well as other problems. Therefore, more straightforward techniques for ROS measurement, particularly the use of direct “real time” or in vivo detections of ROS with more stable and specific probes, are highly desirable, which include chemiluminescence and fluorescence.

1.9.3.3 Chemiluminescence

Chemiluminescence (CL) is the light emitted from chemical reactions, such as reaction between CL probes and ROS. The most common CL probes for ROS detection are luminol and lucigenin. Luminol can react with a variety of ROS such as H₂O₂, O₂•−, and •OH, while lucigenin seems to be more specific to O₂•− (147). Both probes can be used
for intra- or extracellular ROS detection. However, most recent studies have shown that luminol seems to work better for intracellular ROS detection, while lucigenin seems to be more suited for extracellular ROS detection (36). With CL probes, it is possible to directly monitor the ROS signal in real time and in vivo. However, several limitations should be mentioned: 1) Both luminol and lucigenin lack sufficient specificity for particular ROS (147); 2) The loading of these CL probes into cells is not easy (36); 3) Generally speaking, the sensitivity of CL probes is relatively low, although other probes such as L-012 (a luminol derivative compound) have recently been shown to have an enhanced sensitivity to ROS (99); 4) Luminol and lucigenin amplified CL can be greatly influenced by drugs such as penicillin and metals such as Cu^{2+} and Co^{2+} (188).

1.9.3.4 Fluorescence

When the excited electrons return from the lowest orbital of the first singlet state (S₁) to the ground state (S₀), the energy associated with these electrons is emitted in the form of light with a specific range of wavelengths, resulting in a process called fluorescence. In the past decade, fluorescent probes have been widely used in the detection of ROS and RNS in biological systems. Compared to CL probes, fluorescent probes seem to prevail for several reasons: 1) they are more sensitive and specific to ROS and RNS; 2) most fluorescent probes can be easily loaded into the cells or tissues; 3) commercially, they are more available (248). Compared to spin trapping techniques, fluorescent probes are more stable and require much lower concentration of the dyes, thus avoiding possible toxicity or artifacts. Therefore, many fluorescent probes are ideal for monitoring intracellular
ROS or RNS in intact living cells or tissues (248). The details of these probes will be further discussed in chapter 6 of this dissertation.

1.9.3.5 Extracellular ROS detection

CL probes such as lucigenin can be used to detect ROS release from tissues or cells (36). Some fluorescent probes such as cell-impermeable Amplex Red, can also be used as extracellular H$_2$O$_2$ detection (212). However, the cytochrome c assay may be the most common method for extracellular ROS (specific for O$_2^•−$) detection (117,143,144,247) due to its high sensitivity and convenience. However, none of these probes are entirely specific to ROS, as it is possible that other substances can oxidize or reduce them. Therefore, these probes should be applied with corresponding antioxidants for further confirmation (247,248).

1.9.4 Unsolved issues regarding the role of ROS in stressed skeletal muscle

Many unsolved important problems and unknown mechanisms remain in this area of research. These include: 1) the lack of direct evidence for ROS produced in both heat stress and hypoxia in skeletal muscle; 2) the lack of evidence of the molecular source of ROS during heat stress in skeletal muscle; 3) the lack of sufficient attention to the role of lipoxygenase pathways in ROS formation in skeletal muscle. Therefore, my dissertation research will be focused on these major points as expressed in each specific aim in the next section. Furthermore, I will also summarize the most common fluorescent probes for ROS and RNS detection in the field as well as their applications in living tissues with corresponding experimental techniques.
1.10 Specific aims in this dissertation

Specific aim 1: To test the hypothesis that heat stress directly stimulates intra- and extracellular ROS formation in skeletal muscle.

Rationale 1: Skeletal muscles have been shown to generate considerable ROS in both normal and fatigued conditions, suggesting an important role of ROS in muscle function (181,182). Temperature elevations have potent effects on many biological systems, and they may have particular relevance to skeletal muscles because these muscles are biological heat generators (27,28). Although heat shock proteins are upregulated in skeletal muscle during heavy exercise, the direct relationship between heat and ROS generation is still unknown. Therefore, this aim is designed to look for direct evidence for heat-induced ROS formation in skeletal muscle.

Specific aim 2: To test the hypothesis that ROS release is dependent on mitochondrial complex activity or NADPH oxidases, and this release is mediated by the function of anion channels.

Rationale 2: Mitochondria are believed to be the most plausible ROS generators under a variety of pathophysiological conditions (44). However, it is not clear whether mitochondria can significantly contribute to extracellular $O_2^{•−}$ release in intact cells, both in normal and heat-stressed conditions. To do so would not only require a mechanism to transport $O_2^{•−}$ across the mitochondrial membrane but also a mechanism for $O_2^{•−}$ exiting
through the plasma membrane. Previous studies have shown that anion channels can mediate $O_2^{\bullet-}$ release from either mitochondrial membrane or isolated red blood cells (139,230). Thus, it is possible that $O_2^{\bullet-}$ may exit the mitochondria and cytosol through anion channels.

The membrane-associated NADPH oxidases are another potential source of ROS (63,133,229). These enzymes may either require anion channels to transport $O_2^{\bullet-}$ or directly reduce extracellular oxygen into $O_2^{\bullet-}$ (197). However, their role in ROS release from both normal and stressed skeletal muscle is not clear. This would require a specific blocker of these enzymes to test their role in ROS formation and release.

Specific aim 3: To test the hypothesis that $O_2^{\bullet-}$ release at rest and in heat exposure is associated with formation and metabolism of arachidonic acid (AA).

Objective 3a: To test the hypothesis that $O_2^{\bullet-}$ release is related to activity of phospholipase A$_2$ (PLA$_2$), one of the major upstream enzymes of AA metabolism.

Rationale 3a: Previous research has demonstrated that ROS formation can be linked to the activity of PLA$_2$ in skeletal muscle (157), although the mechanism is not fully understood. Further research has shown that exposure of living cells to AA alone can cause sufficient ROS generation (240), suggesting that PLA$_2$ may up-regulate this
process. Therefore, it is necessary to test the role of PLA₂ in AA-related ROS formation in heat stress.

Objective 3b: To test the hypothesis that increased O₂•⁻ release depends on the stimulation of nitric oxide synthase (NOS).

Rationale 3b: Recent research has suggested that AA release is related to NOS-dependent ONOO⁻ formation (87). This is because ONOO⁻ has been shown to be a potent stimulator of PLA₂ activity (87). An effective way to impede ONOO⁻ formation is to use NOS inhibitors, and thus the activation of PLA₂ by ONOO⁻ and the corresponding PLA₂-dependent ROS release would presumably be reduced.

Objective 3c: To test the hypothesis that the downstream pathway of extracellular O₂•⁻ release depends on the activities of cytochrome P450-dependent monooxygenase (P450), cyclooxygenase (COX), and/or lipoxygenase (LOX).

Rationale 3c: The activities of P450 (20,170), COX (164,236), and LOX (138,240) are three primary downstream pathways of AA metabolism. Previous studies have shown that these pathways are related to ROS formation in various cells or tissues. However, their role regarding ROS formation in stressed skeletal muscle has not been determined. Therefore, with corresponding blockers for these enzymes, the degree to which these
pathways contribute to the extracellular ROS release from skeletal muscle can be discerned.

Objective 3d: To test the hypothesis that the source of $O_2^{\cdot-}$ release is independent of the mechanism of intracellular $O_2^{\cdot-}$ formation.

Rationale 3d: It is possible that the extracellular $O_2^{\cdot-}$ release arises from the spillover of intracellular $O_2^{\cdot-}$ generation. However, there is always a certain degree of difficulty in the transport of charged $O_2^{\cdot-}$ across biological membranes. Therefore, it is possible that intra- and extracellular ROS may be generated from different mechanisms, which will be tested with corresponding intra- and extracellular probes of $O_2^{\cdot-}$.

Specific aim 4: To test the hypothesis that hypoxia directly stimulates ROS formation in skeletal muscle.

Rationale 4: ROS are commonly believed to be generated during reoxygenation after hypoxia, since hypoxia may cause a significant accumulation of reducing compounds in mitochondria including NADH, FADH and reduced CoQ (55,231). Whether ROS are directly generated during hypoxia in skeletal muscle remains controversial. Previous studies have shown that antioxidants have protective roles on the function of skeletal muscle, suggesting a possibility of ROS formation in hypoxia (150). However, the direct evidence of hypoxia-induced ROS generation in skeletal muscle is not available.
Therefore, ROS-sensitive fluorescence probes will be used to load tissues, and
fluorescence signals will be detected by a four-channel tissue fluorometer.
Corresponding antioxidants will be also used to specify ROS signals (206,247).
CHAPTER 2

INTRA- AND EXTRACELLULAR MEASUREMENT OF REACTIVE OXYGEN SPECIES PRODUCED DURING HEAT STRESS IN DIAPHRAGM

2.1 Abstract

Skeletal muscles are exposed to increased temperatures during intense exercise, particularly in high environmental temperatures. We hypothesized that heat may directly stimulate the reactive oxygen species (ROS) formation in diaphragm (one kind of skeletal muscle) and thus potentially play a role in contractile and metabolic activity. Laser scan confocal microscopy was used to study the conversion of hydroethidine (HE, a probe for intracellular ROS) to ethidium (ET) in mouse diaphragm. During a 30 min period, heat (42°C) increased ET fluorescence by 24±4%; whereas in control (37°C) fluorescence decreased by 8±1% compared to baseline (P<0.001). The superoxide scavenger Tiron (10mM) abolished the rise in intracellular fluorescence; whereas extracellular superoxide dismutase (SOD, 5000 U/ml) had no significant effect. Reduction of oxidized cytochrome c (cyt c) was used to detect extracellular ROS in rat diaphragm. After 45 min, 53±7 nmole of cyt c/g dry wt /ml was reduced in heat compared to 22±13 nmole/g/ml in controls (P<0.001). SOD decreased cyt c reduction in heat to control
levels. The results suggest that heat stress stimulates intracellular and extracellular superoxide production, which may contribute to the physiologic responses to severe exercise or the pathology of heat shock.

2.2 Introduction

Reactive oxygen species (ROS) play an important role in many biological systems. ROS formation is closely related to the body's response to infection, ischemia-reperfusion, heavy metal and ethanol toxicity, as well as to many other conditions (62,152). It is also believed to play important roles in normal cell signaling events (53,71). Skeletal muscles have been shown to generate considerable ROS under normal conditions. For example, in vitro rat diaphragm produces moderate levels of intracellular (157,182) and extracellular ROS (181) at rest, and production increases markedly with repetitive muscle contractions (117,181).

In this study, we test the hypothesis that increased ROS production in skeletal muscles is also associated with exposure to mild heat stress. Temperature elevations have potent effects on nearly all biological systems; however, they may have particular relevance to skeletal muscles because these muscles act as natural heat generators during exercise. For example, in rats exercised to exhaustion, average limb muscle temperatures can reach 44°C and core temperatures can reach 42°C (28). These temperatures would result in heat shock or a stress response in most muscle cells, causing upregulation of stress proteins (152,209). Although stress proteins are upregulated in skeletal muscle during
extreme exercise (107,209), the relationship between ROS generation, exercise and heat is still unknown.

Results of the current study demonstrate that markedly increased ROS formation, particularly superoxide ($\text{O}_2^\cdot$), is a natural response of the myocyte to heat exposure at temperatures known to be physiologically relevant to exercise. Furthermore, these signals are shown to be blocked by antioxidants specific for $\text{O}_2^\cdot$. The findings may provide insight into understanding the physiologic and pathophysiologic responses to heat exposure in exercising or nonexercising skeletal muscle.

2.3 Methods

2.3.1 Surgical procedures and the diaphragm strip preparation
Male, adult Sprague-Dawley rats (300-500g) and Swiss-Webster mice (20-30g) were used according to the animal care guidelines at the Ohio State University. The animals were anesthetized with intraperitoneal pentobarbital sodium (~ 40mg/kg), tracheotomized and mechanically ventilated. For rat studies, the animals were first heparinized (~500 U/kg) via direct injection into the jugular vein, and the diaphragm was then perfused and completely cleared of blood by retrograde perfusion with oxygenated Ringer's solution from the inferior vena cava. This was done to eliminate possible blood and serum contamination during the cytochrome c (cyt c) measurements. Four diaphragm muscle strips (approximately 0.9 cm wide for rats, 0.5 cm for mice) were dissected out with the corresponding rib attachment and central tendon, placed in Ringer’s solution (mEq/l: 21
NaHCO₃, 0.9 MgCl₂, 1.2 NaHPO₄, 0.9 Na₂SO₄, 2.25 CaCl₂, 5.9 KCl, 109 NaCl and 2.07 g/l glucose with 12 μM d- tubocurarine), and bubbled with 95% O₂ and 5% CO₂.

2.3.2 Extracellular ROS studies

The cyt c assay was used to measure extracellular O₂⁻⁻ release from rat diaphragm, employing a combination of methods as described by Margoliash et al., (143), Reid et al. (181) and Kolbeck et al. (117). O₂⁻⁻ can reduce cyt c through a one-electron transfer reaction, resulting in an increase of absorbance peak at 550 nm, with an extinction coefficient of 18.5 x 10³ M⁻¹ cm⁻¹ (117,126,144). In order to test whether O₂⁻⁻ is released from tissue during heat stress, each rat diaphragm strip was loaded into a 2.9 ml water-jacketed mini-bath (Radnoti, Monrovia, CA) filled with oxygenated Ringer’s solution and 5 μM cyt c (Acros Organics). A tension of 2 g was placed on each strip, which in previous studies was shown to approximate optimum length for the size of the muscle utilized and the inherent weight of the transducer attachments for the muscle (60). The experiments were performed in a darkened room (preventing possible photobleaching of cyt c). The measurement of cyt c reduction was obtained by taking the difference between the peak absorbance at 550 nm and the average of the peaks at wavelengths of 540 nm and 560 nm, as described by Kolbeck et al. (117). This procedure avoided errors in measurement due to non-cyt c specific increases in absorbance during these experiments.

2.3.2.1 Heat treatment only

Two muscle strips from the same animal were studied simultaneously, each fitted into
separate mini-baths. After a 20 min equilibration at 37°C, one bath was kept at 37°C (control); the other was heated to 42°C (heat) for 45 min. The reduction state of cyt c in the baths was measured by removing 1 ml of the bath solution and immediately transferring it to a cuvette and measuring absorbance using a diode-array UV-visible spectrophotometer (HP 8452A, Hewlett-Packard). The cuvette sample was then immediately returned to the bath. Measurements were made every 15 min.

2.3.2.2 Heat treatment plus antioxidants

To test for the specificity of the cyt c assay for O$_2^•^-$ release, heat and control experiments were repeated with strips pre-incubated for 30 min on ice with 5000 U/ml superoxide dismutase (SOD, Sigma) or catalase (1000 U/ml, Sigma). SOD is an effective O$_2^•^-$ scavenger. Catalase, a hydrogen peroxide (H$_2$O$_2$) scavenger, was used to test for possible H$_2$O$_2$ release, since H$_2$O$_2$ can oxidize reduced cyt c and therefore potentially affect the quantification of cyt c reduction (120,232).

2.3.2.3 Controls for the cyt c assay

These experiments were performed to answer a variety of questions related to the cyt c assay at both 37°C and 42°C over the 45 min experimental period. Every attempt was made to mimic the biological conditions studied, as described above. The following issues were studied: a) autoreduction of cyt c and b) cyt c chemical interactions with SOD, 1,2-dihydroxybenzene-3,5-disulfonate (Tiron, Aldrich) and catalase. An additional experiment was performed to determine the release of cyt c from the tissue in heat by
soaking the muscle in oxygenated Ringer’s solution, without cyt c, prior to the 45 min heat and control exposure.

2.3.3 Intracellular ROS studies-confocal microscopy

The hydroethidine/ethidium fluorescent probe was used to detect intracellular ROS production in mouse hemi-diaphragms. Hydroethidine (HE, Molecular Probes) is a non-charged fluorescent probe specifically sensitive to $O_2^{•−}$, peroxynitrite ($\text{ONOO}^{−}$) and hydroxyl radical ($^{•}\text{OH}$) but not to $\text{H}_2\text{O}_2$ (3,31). HE stock was made in N,N-dimethylacetamide (Acros Organics). In response to ROS, HE is dehydrogenated, resulting in the formation of ethidium (ET). ET is positively charged and has better cellular retention and stability compared to HE. For this reason, ET formation was chosen as an indicator of ROS production, which is a common method when using this probe (3,31,157). Mouse diaphragms were used because preliminary studies demonstrated better loading of HE in mouse compared to rat. Furthermore, the mouse diaphragm is considerably thinner, compared to the rat diaphragm, which improves the working distance, the quality and the resolution of the confocal fluorescence imaging analysis. Muscle strips were loaded with 44 µM HE and 4 mM actin-myosin complex blocker, 2,3-butanedione monoxime (BDM, Acros Organics), in Ringer’s solution, in the dark, on ice, for one hour. BDM was used throughout the experiment to block movement of the tissue during heat exposure. When tissue movement exceeded 10% of the size of the field in any direction during data collection (based on visual landmarks and pattern recognition), the data were discarded. In addition, vertical artifacts due to any residual BDM-insensitive movements were minimized by selecting an appropriately thick optical
slice (i.e. ~ 10 μm). In some experiments, z-sectioning analysis done before and after heat experiments indicated that the intensity of the ET signal after heat was invariably greater than that for any of the vertical optical slices before heat (data not shown). This strongly supports that any changes obtained were due to heat and did not involve vertical movement artifact. Following incubation, the muscles were washed for 10-15 min with fresh oxygenated Ringer’s, placed in a flow-through tissue chamber (Biophysica, Ontario, Canada), stretched carefully with wires attached to magnetic holders to approximately 120% of relaxed length and put on the stage of a Laser Scan Confocal and Spatial Imaging Analysis System (Zeiss 410, Carl Zeiss, Germany). An inverted confocal microscope was used and the tissue was excited by the appropriate lasers through a # 0.0 coverslip (0.11-0.17 mm thick) forming the bottom of the chamber. After a 15 min equilibration at 37°C using constant superfusion of heated, oxygenated Ringer’s (with 4 mM BDM), measurements of ET fluorescence were made every 5 min. Although loss of HE fluorescence was also measured (excitation: 364 nm, emission: 480 nm ± 30 nm), our preliminary data showed that the non-polar HE probe had a relatively lower cellular retention than ET, its fluorescence was more easily influenced by environmental solutions (157), and the background from NADH within the myocyte fluoresces in the same emission range as HE (74). The setup for laser scan confocal imaging of ROS was the following: argon/krypton laser power: 10; objective: 63x with 1.5 mm working distance; numerical aperture: 0.75; ET excitation: 568 nm; ET emission: long pass (LP) 590 nm; contrast: 180-250; brightness: 9500-9999; pinhole: 150 (i.e. its corresponding optical section has a thickness of 9.75 μm). The emitted fluorescent signal captured by a photomultiplier tube was presented as an image of 512 x 512 pixels on the laser scan
microscope (LSM) computer monitor. The LSM 410 software was used to analyze the fluorescence image, representing a small square of ~ 0.04 mm² of the tissue. Most fields contained portions of approximately 4-7 myocytes. The average fluorescence over the entire field was collected and the averaged image of 4 consecutive scans of the same field was saved.

A number of experiments, which paralleled the cyt c assay experiments previously described, were performed as described below.

2.3.3.1 Heat treatment only

In these experiments, the muscle strips were superfused with oxygenated Ringer’s solution and 4 mM BDM at 37°C for 5 min and then the stage temperature was increased to 42°C for 30 min. Temperatures were measured to within 0.1°C directly in the superfusate bathing the tissue, using a miniature thermistor (YSI 4610, Yellow Springs). Control muscle strips were treated identically, except that the superfusate was maintained at 37°C throughout the experiments.

2.3.3.2 Heat plus antioxidant treatment

Tiron (10 mM, a membrane-permeable scavenger of O₂⁻ or SOD (5000 U/ml, non-permeable to membranes) was added to 37°C perfusate, 15 min before the measurement in both heat and control experiments in order to let the tissues be fully equilibrated with these antioxidants.
2.3.3.3 Nuclear localization of ET fluorescence

In order to identify whether ET fluorescence is concentrated within the cell nucleus or cytoplasm, 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI, 1 µg/ml, Sigma) was used as a fluorescent marker for the nucleus. All other conditions were the same as in section (a). LSM overlay image analysis was used to correlate regions of ET fluorescence (excitation: 568 nm, emission: LP 590 nm) overlapping with regions of DAPI fluorescence (excitation: 364 nm, emission: 480 nm ± 30 nm) at the control temperature.

2.3.4 In vitro tests of the ET fluorescence assay

Biochemical controls were used to test the specificity of the ET fluorescence assay for heat-induced reactive oxygen using a standard cuvette fluorometer (Model 5050, Perkin Elmer). These tests were repeated with reagents used in the tissue experiments.

2.3.5 Statistical analysis

Values are expressed as means ± SE. Data were analyzed with SAS JMP (SAS Institute Inc., Cary, NC) using 2-way ANOVA with heat treatment and time being the factors of interest and the experimental animal treated as a random variable. Post hoc contrasts were used to determine differences in mean values at specific time points and between treatments. $P < 0.05$ was considered statistically significant.
2.4 Results

2.4.1 $O_2^{•−}$ release from tissues in heat stress

As shown in Fig. 2.1, heat exposure caused significant increases in cyt c reduction, as early as 15 min into the test. After 45 min exposure to heat, a total of $53 \pm 7$ nmoles cyt c /g dry wt/ml solution was reduced compared to control ($22 \pm 13$ nmoles/g/ml, N=8, P < 0.001).

Fig. 2.2A illustrates that extracellular SOD treatment blocked the influence of heat treatment on $O_2^{•−}$ production. After 45 min, the SOD- and heat-treated muscles demonstrated a total cyt c reduction ($21 \pm 8$ nmoles/g/ml) which resembled control, i.e. SOD- and non-heat- treated diaphragm ($13 \pm 7$ nmoles/gm/ml). Furthermore, there were no significant differences between SOD-treated control and SOD-heated muscle at any time point (N=5). As will be demonstrated later, Tiron caused direct reduction of cyt c, independent of the presence of diaphragm tissue, thus prohibiting its use in evaluating mechanisms of extracellular cyt c reduction by muscle tissue.

The catalase experiments are illustrated in Fig. 2.2B. After the 45 min exposure to heat stress, there were no significant differences between catalase-treated ($51 \pm 5$ nmoles/g/ml) and non-catalase-treated ($63 \pm 7$ nmoles/g/ml) tissues (N=6 from 3 animals), suggesting that there was no significant $\text{H}_2\text{O}_2$ released from these diaphragms in heat stress.
2.4.2 Biochemical controls for the cyt c assay (Fig. 2.3)

Potential autoreduction of cyt c was tested by exposing 5 µM cyt c in oxygenated Ringer’s solution to 42°C and 37°C. After 45 min, cyt c was reduced similarly in both heat-treated (0.09 ± 0.08 nmoles/ml) and control (0.07 ± 0.03 nmoles/ml, n.s. N=5), accounting for < 3% of the total cyt c in solution. SOD treatment (5000 U/ml) resulted in a reduction of <5% and catalase (1000 U/ml) < 18% of the total cyt c in the solution, with no additional effects of heat treatment. In contrast, 10 mM Tiron resulted in rapid and immediate reduction of cyt c, reducing the cyt c at both 37°C (4.8 ± 0.19 nmoles/ml, N=3) and 42°C (4.1 ± 0.13 nmoles/ml, N=3) and accounting for > 80% of the total cyt c available for reduction in solution.

2.4.3 Intracellular ROS formation in heat stress (Fig. 2.4)

As shown in Fig. 2.4A and 2.4B, under confocal microscopy, ET fluorescence increased markedly in heat-treated mouse diaphragm over 30 min (increase in red), with no significant increase in ET fluorescence in control tissue at 37°C (Fig. 2.4C and 2.4D). Typically, the ET fluorescence was heterogeneous, both within individual fibers and within the nuclei and cytosol of the myocytes. Grouped data are shown in Fig. 2.5A. After a 5 min pre-heat measurement (in which ET fluorescence was basically constant), rapid and significant increases in ET fluorescence were observed within 15 min, increasing 24 ± 4% of baseline by 30 min. In contrast, control (37°C) fluorescence decreased 8 ± 1% of baseline during the same period (N= 6, P<0.001). It is worth noting that the observed increases in ET fluorescence represented an underestimate of the change, because the entire field was used in analysis rather than individual myocytes. In
all experiments, considerable background ET fluorescence was observed in the baseline images. This presumably was due to endogenous ROS formation in resting tissue (36).

2.4.4 Effects of antioxidants on intracellular ROS

Treatment with Tiron (10mM, Fig. 2.5B), a $\text{O}_2^{**}$ scavenger accessible to the intracellular compartment, completely removed the effect of heat on ET fluorescence in diaphragms. Furthermore, it reduced the ET fluorescence by $7 \pm 6\%$ and $12 \pm 3\%$ in heated and control tissues, respectively (n.s., N=3). In contrast, continuous SOD treatment (5000 U/ml) in the superfusate did not decrease the ET fluorescence observed during exposure to heat (Fig. 2.5C), suggesting that this ROS signal originated from the intracellular compartment.

2.4.5 Localization of ET fluorescence

As illustrated in Fig. 4E and 4F, double staining during 37°C with DAPI and HE resulted in co-localization of DAPI (blue fluorescence, Fig. 2.4E) with ET (red fluorescence, Fig. 2.4F). We noticed that more than 90% of the blue dots (nuclei) superimposed on the red dots in these experiments. Taken together, it is evident that the ET signal localizes in both the nucleus and cytoplasm, although the intensity of the signal in the nuclei is higher than in the cytosol. Whether ET and DAPI also co-localized in the mitochondria was not distinguishable at these magnifications. Note, as compared to DAPI, which has a relatively high quantum yield, HE blue fluorescence is almost undetectable at the laser and LSM settings used to detect DAPI (contrast=213), and therefore did not contribute to the DAPI-blue fluorescence.
2.4.6 Biochemical controls for confocal microscopy in vitro

Control experiments demonstrated that 1) heat does not significantly affect the spontaneous conversion of HE to ET; 2) Tiron does not affect HE or ET fluorescence; 3) BDM causes < 9% decrease by HE baseline fluorescence without significant conversion from HE to ET, and 4) BDM and Tiron together decrease HE baseline fluorescence by < 9%, but without changing ET fluorescence (data not shown).

2.5 Discussion

These results demonstrate that exposure to heat stress results in rapid and significant elevations in extracellular and intracellular reactive oxygen production. Although the levels of heat treatment used were within the physiologic range measured in exercising rat muscle (28) and in humans during exercise in heated environments (204), exposure to this temperature is sufficient to up-regulate heat shock proteins (209), and therefore can appropriately be categorized as heat stress. Treatment with relevant O$_2^\cdot$ scavengers strongly suggests that the primary or initial species generated during heat stress in muscle is O$_2^\cdot$, as measured by both extracellular and intracellular probes. The direct production of O$_2^\cdot$ during heat may have implications with regard to mechanical, pathological and cell signaling responses to extreme exercise and fatigue as well as responses to exercise in elevated environmental temperatures.

2.5.1 Measurement of O$_2^\cdot$ release from diaphragms in heat stress

Previous studies by Reid et al. (181) and Kolbeck et al. (117) have used the cyt c assay to
demonstrate $O_2^{•−}$ generation in resting muscle and have shown marked increases in production during muscle stimulation. The present work extends these findings to conditions of heat stress, suggesting the possibility that extracellular ROS formation is a generalized response to a number of stresses to muscle, which may be related to exercise. Cyt c is used as an electron acceptor in these studies and theoretically, any electron donor secreted from the tissue with the appropriate redox potential, could reduce cyt c. However, the inhibition of the heat induced reduction of cyt c by SOD provides relative certainty that the assay measured $O_2^{•−}$ and not another unknown substance released during heat. The cyt c approach has been used successfully in many other biological models, including but not limited to brain injury during hypoxia (67), dopamine oxidation in the nervous system (110), and menadione-stimulated pyridine nucleotide oxidase activity from neutrophil membranes (160). The large molecular size of cyt c (12.4 kDa) prevents its penetration through the sarcolemma, making it an exclusively extracellular probe (181). Additionally, in agreement with previous studies (181), we confirmed that it is remarkably stable in physiologic, oxygenated buffers, as well as in the presence of SOD and catalase solutions.

2.5.2 Potential sources of ROS production in heat stress

A number of potential cellular sources for ROS have been proposed in skeletal muscle. Although mitochondria are strong potential candidates, other sources are possible. For example, the diaphragm has a very large capillary bed, which has been estimated to be at least twice that of a comparable muscle fiber population (108). Therefore, the isolated muscle has a large amount of capillary endothelium. Endothelial cells are now
understood to be potent potential sources of ROS production via NADH and/or NADPH oxidases on their cell membranes (56,153). Reactive oxygen production may play a role in vascular responses to shear stress and other stimuli, which may be important in local vascular regulation (56). Therefore, the extracellular signal observed in these experiments in response to heat stress, could conceivably have arisen from endothelial sources as well as myocyte sources. O$_2$– is believed to be capable of diffusing across cell membranes, and it has been postulated that extracellular O$_2$– produced by skeletal muscle may exit the myocyte directly by diffusion (181,182). This was based largely on the influence of extracellular antioxidants, such as SOD on contractile properties, but also on the inhibition of dichlorodihydrofluoroscein oxidation by extracellular SOD (182).

In contrast, the intracellular ROS production, as indicated by HE/ET fluorescence, was clearly localized within the myocyte. It is possible that the myocyte may also contain cytosolic or membrane-bound NADPH or NADH oxidoreductases; however, these have not been identified. Furthermore, xanthine oxidase (56), nitric oxide synthase (173) or arachidonic acid metabolism (51) have also been presented as potential sources of ROS in skeletal muscle and other tissues. It is possible that the activity of these enzymes may be uniquely affected by heat and/or the influx of intracellular Ca$^{2+}$ during heat stress; however, their role has yet to be identified in muscle.

A likely source of elevated ROS production in heat is the mitochondrion. Mitochondria are believed to produce basal levels of ROS in the form of single electron leakage to O$_2$ during normal metabolism (44). This can increase dramatically under conditions in
which the mitochondria are damaged or exposed to certain toxic conditions. The response of the mitochondria to heat is only partially understood. In the 1970’s, a number of studies evaluated the relationships between temperature, muscle function and oxygen consumption. O$_2$ consumption was found to increase markedly, immediately after exercise. Although there are a number of potential mechanisms for this phenomenon, it has been proposed that it may in part be related to increased temperature (28). At high body temperature, skeletal muscles exhibit a lower level of phosphorylation efficiency and a higher respiratory rate (27,28). Furthermore, higher temperatures have a significant effect on mitochondrial function, although this has not been studied extensively. For example, from 25°C to 45°C, as the mitochondrial oxygen consumption increases ~ 2.5 fold, mitochondria-specific ATPase activity increases ~ 4 fold (27). Finally, elevated temperatures have been shown to result in increased ATP utilization and creatine phosphate depletion (69). The mechanisms for these changes in mitochondrial function and metabolism are not known, but they may be consistent with conditions that promote oxidant production (161). Alternatively, they may reflect the effects of oxidant production on mitochondrial function or both.

Nethery et al. have recently demonstrated that phospholipase A$_2$ activation appears to be a necessary step in the increase of skeletal muscle reactive oxygen formation during muscle stimulation (157). The activation of phospholipases may result in reactive oxygen formation by first generating arachidonic acid from available membrane substrates. Arachidonic acid may then interfere directly with electron transport, produce
ROS via cyclooxygenase pathways or by direct interaction with the mitochondrial electron transport chain (51).

Another potential source of the increase in both the extracellular and intracellular ROS measurements is the influence of heat on *NO production. Since *NO is known to react rapidly with $O_2^{•−}$ to form $ONOO^−$ and other down stream metabolites, any change in NOS activity could affect the superoxide signal measured by these assays. The current experiment evaluated responses over a period of one hour or less, making changes in *NO production due to new NOS gene expression unlikely. However, it is highly probable that NOS activity was directly augmented by increased temperature. For example, Venturini et al. have demonstrated that NOS I and NOS II (i.e. nNOS and iNOS) increase their activity \textit{in vitro} by nearly 100% between temperatures of 37°C and 42°C (233). Such an elevation in *NO would presumably diminish the extracellular cyt c reduction, because any additional $ONOO^−$ formed would oxidize cyt c (43). Therefore, our overall conclusions in this paper regarding the effects of heat exposure on extracellular $O_2^{•−}$ release are qualitatively correct, though possibly underestimated. The effects on intracellular HE oxidation are more complex since both $O_2^{•−}$ and $ONOO^−$ oxidize HE to ET (3) and both species would be inhibited by Tiron administration. Therefore, it is possible that some of the increased intracellular ET signal we observed could be due to a contribution of augmented NOS activity from $ONOO^−$ in heat. Further experiments will be required to address this complex problem.
2.5.3 H$_2$O$_2$ release from the tissue

Since H$_2$O$_2$ oxidizes reduced cyt c (120,232), if an appreciable amount of H$_2$O$_2$ had been released into the extracellular space, before or during heat stress, we would have expected an increase in the net reduction of cyt c with catalase treatment during heat stress. Since this did not happen, we speculate that endogenous intracellular H$_2$O$_2$ scavengers such as catalase, the glutathione peroxidase system or the thioredoxin peroxidase system, were sufficient to remove H$_2$O$_2$ before it was able to diffuse out of the tissue in significant quantities. Why O$_2^-$ was not scavenged by intracellular SOD is a point of some speculation and may reflect unique pathways of O$_2^-$ excretion from the cell, which have yet to be fully identified.

2.5.4 Measurement of intracellular O$_2^-$ formation in heat stress

HE was chosen as the fluorescent probe of choice in these experiments, largely because preliminary experiments using more common probes, such as derivatives of dichlorofluorescein, proved unsuitable at elevated temperatures. At 37°C and particularly at 42°C, the extremely poor retention of these probes made it impractical and perhaps impossible for use in studies of heat stress (data not shown). HE, an uncharged molecule, can enter and stain cells with little or no toxicity. It is easily reduced by O$_2^-$, ONOO$^-$ and ·OH to form ET, a polar molecule with a positive charge, which is subsequently trapped inside the cell. The HE/ET probe has been used as a sensitive O$_2^-$ indicator in studies using endothelial cells (40), cerebellar granule neurons (31,195), tumor cells (30), lungs (3) and inflammatory cells (219). Recently, Nethery et al.(157)
demonstrated its use in perfused diaphragmatic muscle, where it was measured by extraction after the experiment. In this study we employed confocal microscopy which is very useful to observe changes in fluorescence in real time and to provide direct evidence of intracellular localization. The confocal image represents information originating from the optical slice in the plane of focus with unfocused images from other planes excluded, resulting in the improvement of the image’s resolution across the cell. In contrast to studies of the cyt c probe, there was no chemical interaction between the cell-permeable $O_2^{\bullet-}$ scavenger, Tiron, and the HE/ET probe, thus allowing the use of this antioxidant to block the intracellular signal. The signal, however, was not inhibited by extracellular application of SOD, suggesting an intracellular localization of $O_2^{\bullet-}$ generation within the myocytes. Although $H_2O_2$ must also be formed as the result of the reaction between Tiron and $O_2^{\bullet-}$ (86), HE is relatively insensitive to $H_2O_2$ and thus, the assay is directed largely to $O_2^{\bullet-}$, ONOO$^-$ and $^\bullet OH$ (3,31,157). The DAPI, nuclear labeling technique (Fig. 2.4E), provided direct evidence for the localization of the ET signal (Fig. 2.4F), in part, within the nuclei. However, additional ET was always seen in the cytoplasm or perhaps the mitochondria. Localization of ET within the nucleus is not surprising because the positively charged molecule is a well-known DNA stain and no doubt diffuses into the nucleus regardless of the original source of its intracellular generation. However, further work will be required to identify the intracellular sites of ROS production in heat stress.

2.5.5 Functional implications of heat-induced ROS production

Contractile properties of skeletal muscle at temperatures between 37°C and 42°C have not been studied extensively. Prezant et al. (176) demonstrated that force development
and twitch kinetics are relatively well preserved at temperatures of 41°C compared to 37°C. Preliminary experiments from our laboratory have demonstrated small but significant reductions in force production at all stimulation frequencies following exposure of *in vitro* diaphragm to 15 min of 42°C heat stress (unpublished observations). These effects are sustained over three hours after the heat stimulus. Another important contractile response to heat stress involves the increases in passive tension and rigidity of skeletal muscle. The causes of this phenomenon are not clear, but may involve increases in resting Ca\(^{2+}\) or Ca\(^{2+}\)-independent changes in the molecular conformation of actin-myosin filaments and their interactions with tropomyosin (179). Our results suggest the possibility that some of the contractile responses during heat stress may reflect the influence of low level reactive oxygen production. Reactive oxygen is now believed to play some poorly defined role in depressing muscle function during fatiguing stimulations (60,182,205) through possible oxidative modification of proteins involved with excitation-contraction coupling, the contractile elements or other cell components. Furthermore, reactive oxygen may be playing an important role as a cell signaling agent, upregulating stress induced transcription factors such as nF-κB (200), upregulating stress proteins such as HSP\(_{70}\) (209), upregulating antioxidants such as manganese-SOD (242) and altering glucose uptake and metabolism (19).

We speculate that increased temperature may amplify the reactive oxygen production associated with intense muscle stimulation, thus augmenting the influence of ROS on muscle fatigue and possibly on long-term muscle adaptation and viability.
Fig. 2.1. Effects of heat treatment on extracellular ROS formation. Cyt c reduction by diaphragm during heat stress compared to control conditions.
Fig. 2.2. Effects of antioxidants on extracellular ROS formation. A: cyt c reduction by diaphragm, with and without heat treatment, in the presence of SOD. B: cyt c reduction by diaphragm during heat stress, with and without catalase.
Fig. 2.3. In vitro cyt c experiments in oxygenated Ringer’s solution. Buffer: autoreduction of cyt c; SOD: reduction of cyt c with SOD; Catalase: reduction of cyt c with catalase; Tiron: reduction of cyt c with Tiron.
Fig. 2.4. Intracellular ROS formation in heat stress. Typical ET confocal fluorescent image of mouse diaphragm (A) before and (B) following 30 min of heat stress (42°C). Typical fluorescent ET images in control diaphragm at (C) baseline and (D) following 30 min at 37°C. From (C) to (D), no increase in ET signal was observed; in fact, a slight loss and redistribution of the ET signal was seen after the control period. In (E) and (F), tissues were loaded by both DAPI and HE. (E): DAPI staining in the nuclei of the myocytes. (F): baseline ET produced is localized mainly in DAPI-stained nuclei (see method). (Note: paired A-B, C-D, and E-F represented images from 3 different diaphragms from different mice demonstrating considerable variability in ET baseline signals between diaphragms. For example, differences in contrast between baseline signals in A and C are typical, and in part due to different contrast setups in order to maximize the resolution.)
Fig. 2.5. Grouped data for intracellular ROS formation indicated by changes in ET fluorescence. A: Effects of heat vs. control conditions. B: Same experiment as in A, in the presence of Tiron. C: The effects of SOD treatment on ET fluorescence during heat. The data from heat treatment in C (open circles) is the same data shown in A, for comparison. ±: one data point removed at this time point because of >10% movement of tissue out of the field (N=2).
CHAPTER 3

SOURCES FOR SUPEROXIDE RELEASE: LESSONS FROM BLOCKADE OF ELECTRON TRANSPORT, NADPH OXIDASE AND ANION CHANNELS IN DIAPHRAGM

3.1 Abstract

Isolated diaphragm releases low levels of superoxide ($O_2^{*-}$) at rest and much higher levels during heat stress. The molecular source is unknown. The hypothesis was tested that heat stress stimulates mitochondrial complex activity or NADPH oxidases, resulting in increased $O_2^{*-}$ release. The mitochondria within intact rat diaphragm were inhibited at complex I (amobarbital or rotenone), complex I and II (rotenone + thenoyltrifluoroacetone) or complex III (antimycin A). NADPH oxidases were blocked by diphenyliodonium (DPI). None of these treatments inhibited $O_2^{*-}$ release. Conversely, most blockers stimulated $O_2^{*-}$ release. Since intracellular $O_2^{*-}$ generators require a mechanism for $O_2^{*-}$ transport across the membrane, anion channel blockers, probenecid or 4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid, were also tested. Neither blocker had any inhibitory effect on $O_2^{*-}$ release. These results suggest that $O_2^{*-}$ released from diaphragm is not directly dependent on mitochondrial complex activity and that it is not a reflection of passive diffusion of $O_2^{*-}$ through anion channels. We
speculate that the generator must be a membrane bound system that is not an NADPH oxidase and that it is sensitive to temperature and conditions of “chemical hypoxia” induced by partial or complete mitochondrial inhibition.

3.2 Introduction

Previous research from our laboratory has shown that significant reactive oxygen species (ROS), especially superoxide (O$_2$•−), are released from rat diaphragm in heat stress, which follows a pattern very similar to intracellular O$_2$•− production (247). The molecular source, or organelle, responsible for O$_2$•− release in heat stress, or even at normal temperatures, is unknown. Mitochondria are believed to be one of the possible generators of ROS under many pathophysiological conditions (44). In normal mitochondria, the charge on O$_2$•− presumably precludes its movement across mitochondrial membranes, and it is believed to be largely dismutated by mitochondrial superoxide dismutase (SOD) to hydrogen peroxide (H$_2$O$_2$), which can pass through membranes. However, it is not known whether mitochondria can significantly contribute to extracellular O$_2$•− release in intact cells in normal conditions or in stressed conditions such as heat exposure. To do so would not only require a mechanism to move O$_2$•− across mitochondrial membranes but also a mechanism for exiting the cell through the cell membrane. Anion channels have been shown to play a role in O$_2$•− release from intracellular sources in isolated red blood cells (139) and are believed to be important for mitochondrial O$_2$•− transport (230). Thus, it is possible that O$_2$•− may exit the mitochondrion or cytosol via membrane anion channels.
Another potential source of $\text{O}_2^{**}$ release could be membrane-associated oxidoreductases, e.g., NADPH oxidases (63, 79, 133, 229). NADPH oxidases may not require anion channels for $\text{O}_2^{**}$ release, because it is possible that they transport electrons and reduce $\text{O}_2$ directly on the outside of the membrane (197). The presence of specific NADPH oxidases in skeletal myocyte membranes has not been established, but they are known to be present in endothelial cells (56, 153).

In this study, the following hypotheses were tested: 1) leakage of electrons from the mitochondrial electron transport chain is a primary source of $\text{O}_2^{**}$ release at rest and increased $\text{O}_2^{**}$ release in heat stress. This source can be inhibited by blockers of electron transport; 2) Activation of membrane-associated NADPH oxidases is responsible for increased $\text{O}_2^{**}$ release in heat stress; 3) The release of $\text{O}_2^{**}$ into the extracellular environment from any intracellular source requires open anion channels at the cell and/or mitochondrial membrane.

Current results disprove each of these hypotheses, either directly or indirectly, suggesting that other non-mitochondrial and non-NADPH oxidase sources of $\text{O}_2^{**}$ release are predominant. Surprisingly, drug treatments that interfered with mitochondrial electron transport or NADPH oxidases increased $\text{O}_2^{**}$ release.
3.3 Methods

3.3.1 Diaphragm strip preparation

Male Sprague-Dawley rats (350-450 g) were housed and treated according to approved protocols of The Ohio State University Institutional Laboratory Animal Care and Use Committee. Animals were anesthetized with sodium pentobarbital (~40 mg/kg) or the combination of ketamine (~76 mg/kg) with xylazine (~15 mg/kg), tracheotomized and ventilated with room air. The blood was heparinized (~500 U/kg) by injection through the jugular vein. The diaphragm was cleared of blood by retrograde perfusion, through the inferior vena cava with oxygenated Ringer’s solution, to lower the blood contamination that may affect the cytochrome c (cyt c) assay. The diaphragm was then dissected in oxygenated Ringer’s solution into several muscle strips, each with its corresponding central tendon and rib. These strips were kept in Ringer’s solution (in meq/L: 21 NaHCO₃, 1.0 MgCl₂, 1.2 Na₂HPO₄, 0.9 Na₂SO₄, 2.0 CaCl₂, 5.9 KCl, 121 NaCl; 2.07g/L glucose and 10μM d-tubocurarine) on ice, and bubbled with 95% O₂ – 5% CO₂ prior to the data collection period (247).

3.3.2 Cytochrome c assay

O₂⁻⁻ release from rat diaphragm strips was measured with a cyt c assay as described previously (247). Briefly, cyt c (oxidized) can be easily reduced through one-electron donation from O₂⁻⁻, and we previously demonstrated that the primary source of reduction during heat stress is via O₂⁻⁻, because the signal is essentially completely inhibited by extracellular SOD (247). To minimize possible non-cyt c-specific absorbance
interference, measurement of cyt c reduction was calculated by taking the difference between the peak absorbance at 550 nm and the average of the values at 540 nm and 560 nm (117). The extinction coefficient of cyt c used was $18.5 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (117,143,247).

### 3.3.3 Treatment groups

All diaphragm strips were incubated in oxygenated Ringer’s solution, with or without blockers, for 30 min, on ice. Then, each muscle strip was loaded with ~2 g of tension, to approximate optimum length. Prior to any readings, tissues were equilibrated for 10 min at 37°C in a 3.0 ml water-jacketed tissue bath (Radnoti, Monrovia, CA) filled with Ringer’s solution. Control strips were exposed to 5 μM cyt c solution at 37°C for 45 min, with or without corresponding blockers; heated strips from the same animal were exposed to 5μM cyt c solution for 45 min at 42°C, with or without corresponding blockers. The reduction of cyt c in the bath was monitored by transferring 1 ml of bath solution to a cuvette in a diode-array ultraviolet-visible spectrophotometer (HP 8452A, Hewlett-Packard) for an absorbance measurement every 15 min. After each measurement, the solution was immediately transferred back to the tissue bath.

The concentrations used for each blocking agent were equal to or greater than the highest values reported in the literature for whole tissue experiments. This was often limited by the solubility of the agent and the maximum solvent concentration that could be used without causing significant adverse effects. Blockers used included amobarbital (5 mM, Eli Lilly & Co., Indianapolis, IN), rotenone (50 μM), antimycin A (50 μM), thenoyltrifluoroacetone (1 mM, TTFA), 4,4’-diisothiocyanatostilbene-2,2’-disulfonic
acid (0.5 mM, DIDS), probenecid (1 mM) (all from Sigma, Inc.) and diphenyliodonium (1 mM, DPI, Acros Organics). Stock solutions of most chemicals were made in dimethyl sulfoxide (DMSO, Sigma). Amobarbital and DIDS stocks were made in water, and probenecid stock was prepared in a 1N NaOH solution. In all experiments, DMSO concentrations in tissue baths were kept below the level (<0.8% v/v) that would significantly affect muscle contractility (184). Upon the addition of blockers, the pH of the Ringer’s solution was re-adjusted to 7.4-7.6 in all experiments. Corresponding solvent concentrations were used in all matched control experiments.

Each blocker was pre-tested with the cyt c solution in the absence of tissues to check for independent chemical reactivity with cyt c over time. In most cases, the effects of blocking agents were not obvious (< 10% of the fully reduced cyt c). However, we found that DIDS caused a significant reduction of cyt c (~18-38% of the fully reduced cyt c, depending on DIDS and cyt c concentrations), and thus corresponding adjustments in cyt c concentration were made in DIDS experiments. Nevertheless, all data were corrected based on the measured in vitro chemical reduction of cyt c by respective solvents and blockers.

### 3.3.4 Measurements of oxygen consumption

As will be shown in the results section, some of the blockers had unexpected effects on O$_2^*$ release detected by cyt c reduction. The conditions of two critical blocker experiments in the cyt c assay, including the rotenone series and rotenone plus TTFA series, were repeated to test their effectiveness on the inhibition of mitochondrial electron
transport in the intact tissue, as monitored by oxygen consumption. Specifically, each muscle strip was suspended in a 3 ml reaction chamber with an integrated oxygen electrode unit (Oxygraph System, Hansatech Instruments, UK). The strip was mounted on a plastic frame and loaded with 2 g of tension, to approximate optimum length. To reduce sensitivity so that O$_2$ consumption could be measured in the 95% O$_2$ range, specially designed electrodes were utilized, as provided by Hansatech Instruments. The baths were calibrated and equilibrated to near 95% O$_2$, and closed. Magnetic stirrers were used to avoid diffusion gradients within the baths. Protocols of muscle treatment were identical to those used for the cyt c assay at 37°C. To avoid hypoxia, when bath O$_2$ concentrations dropped below 75-80% O$_2$, the bath chamber was opened, re-equilibrated with 95% O$_2$ and closed before continuing the measurement. In all cases, one control tissue was run simultaneously in parallel to a treated tissue (both from the same animal), and was used to calculate percentage inhibition by the drug. The data were analyzed using the Oxygraph software (Hansatech Instruments, UK). The oxygen consumption rate (nmoles/min/g wet wt) was calculated by averaging the slope of the oxygen concentration decrease in the chamber during 2 min periods every 15 min.

### 3.3.5 Statistical and graphical analysis

In most cases, data were analyzed with multi-way ANOVA using JMP (SAS Institute, Cary, NC) and expressed graphically as means ± SE. The individual “rat” was treated as a random variable with drug-treatment and time being the primary factors of interest. The statistical differences in mean values between treatment and non-treatment groups or between different treatments at specific time points were determined by contrast
procedures (JMP software). Where appropriate, corrections were made for repeated
measures done on separate strips from the same animal. P < 0.05 was considered to be
statistically significant.

Considerable between-group and between-animal differences in responses, independent
of drug treatment, were observed. These may have reflected different basal levels of
ROS production in each animal, varied responses of animal groups to concentrations of
the solvents used in each series (e.g. DMSO ranged from 0 to 0.77% v/v) or other
nonspecific differences in the groups such as their age or length of stay in the vivarium.
Such differences were controlled in the analysis by randomization, by matching solvents
in control strips, and by matching controls and experimental treatments from the same
animal. In addition, to simplify the data, results were summarized in a graphical form as
differences from sham controls in the same animal with corrections for in vitro chemical
reactions.

### 3.4 Results

#### 3.4.1 Complex III inhibition with antimycin A (50 μM)

In all experiments, heat treatment significantly increased cyt c reduction, as previously
reported (247). As a typical example, changes in the absolute cyt c reduction, with and
without antimycin A treatment at the given temperature, are shown in Fig. 3.1. On a
theoretical basis, blockage of complex III should result in the increase of ROS
production, because ubiquinone, the electron carrier to complex III is believed to be a
major source of radical formation (21,226). Therefore, complex III inhibition experiments were performed as a positive control. As shown in Fig. 3.1 and 3.2, antimycin A-treated tissues caused more cyt c reduction than non-drug treated tissues at both 37°C and 42°C, indicating that the variation of O₂⁻ release could be monitored in our system (N=6, P<0.05). Furthermore, this pattern, i.e. the absolute changes of cyt c reduction over time, was typical of most responses to the blockage of mitochondria and NADPH oxidases, which were summarized in Fig. 3.2. Results from all treatments expressed in Fig. 3.2 reflect the change from matched control at the same temperature. For example, the zero delta line (x axis) would be equivalent to a zero difference between drug treatment and non-drug treatment at that temperature.

### 3.4.2 Complex I inhibition with amobarbital (5 mM) or rotenone (50 µM)

As shown in Fig. 3.2, amobarbital significantly increased cyt c reduction at 37°C (N=10, from 7 rats, P<0.01), similar to preceding experiments with complex III inhibition. However, at 42°C, amobarbital had no statistically significant effect on cyt c reduction (N=10 from 7 rats). Rotenone treatment resulted in no significant stimulation of cyt c reduction at either 37°C or 42°C over the time course of the experiment (N=8). This raised the question: was the drug used, such as rotenone, getting to the mitochondria in sufficient concentrations to block electron transport in our experimental conditions. Fig. 3.3 showed that under similar conditions, rotenone significantly reduced the measured O₂ consumption at each time point at normal temperature (N=6 from 5 rats, P<0.05). This working model was further tested to be valid by other experiments showing that the
cyanide-inhibited rate of respiration in this preparation was less than 10% of the oxygen consumption in controls (data not shown).

3.4.3 Combined complex I and complex II inhibition with rotenone (50 \(\mu\)M) plus TTFA (1 mM)

Since mitochondria, in their intact state within the cell, can provide a variety of substrates for electron transport through complex I and/or II, it is necessary to simultaneously block both complex I and II to test the role of mitochondria in \(\text{O}_2^{\bullet-}\) release. Tissues treated with rotenone + TTFA caused more cyt c reduction compared to non-drug treated tissues at both 37°C and 42°C (Fig. 3.2; \(N=6, P<0.05\)), thus exhibiting results similar to those of antimycin A. To test the effectiveness of complex I and II blockade, changes in \(\text{O}_2\) consumption induced by these treatments were measured. Fig. 3.3 showed that rotenone + TTFA caused a greater inhibition of \(\text{O}_2\) consumption than rotenone alone at both 30 min and 45 min at normal temperature (\(N=6\) from 5 rats, \(P<0.05\)).

3.4.4 Membrane-associated NADPH oxidases: inhibition with DPI (1mM)

Similar to preceding experiments, DPI did not block cyt c reduction (Fig. 3.2) and, in fact, promoted its reduction at both 37°C and 42°C (\(N=9, P<0.05\)).

3.4.5 Determination of critical concentrations of cyt c

Additional controls were performed to test whether the collision probability between cyt c (oxidized) and \(\text{O}_2^{\bullet-}\), would be dependent on the concentration of cyt c in the bath. This was done by evaluating the difference between reduction measurements at cyt c
concentrations of 4 vs. 5 μM and 3 vs. 5 μM in paired heat-stressed tissues. At each time point, 3 and 4 μM cyt c data were expressed as % of 5 μM cyt c ± SE (Table 3.1). No significant difference was observed between 4 and 5 μM cyt c in heat stress experiments (N=6 from 3 rats), but significant differences were observed between 3 and 5 μM cyt c (P<0.05 at 30 and 45 min, N=6 from 3 rats). Therefore, the use of 4-5 μM cyt c in these experiments appeared sufficient to ensure that the cyt c was not saturated in the reduced form in all experiments. These experiments were critical, because greater than 20% cyt c reduction was observed by pure chemical interactions alone with 0.5 mM DIDS in subsequent experiments. For these experiments, the cyt c concentration was raised to 10 μM to maintain a sufficient level of oxidized cyt c to react with O$_2^{•-}$.

3.4.6 Inhibition of anion channels with probenecid (1 mM) and DIDS (0.5 mM)

As shown in Fig. 3.4, probenecid had no significant effect on cyt c reduction at 37°C and 42°C (N=6). For DIDS experiments, as mentioned above, 10 μM cyt c was used for ROS detection. Similar to probenecid, DIDS had no significant effect on cyt c reduction at 37°C (N=6). However, at 42°C, a trend of increased inhibition can be observed. This may be related to factors such as animal variation. In half of the rats, DIDS caused a small increase of cyt c reduction, while in the other half, DIDS had an inhibitory effect on cyt c reduction. As a whole, the DIDS-treated data did not show any significant inhibition of O$_2^{•-}$ release from the tissue (N=6, P=0.36 at 30 min and P=0.19 at 45 min). Therefore, O$_2^{•-}$ release did not seem to be dependent on the integrity of anion channels.
across the cellular or mitochondrial membranes when blocked by these agents, used at sufficient concentrations.

3.5 Discussion

These results are consistent with the hypothesis that $O_2^{\cdot-}$, released from the diaphragm, both at rest and in heat stress, does not originate from an intracellular, cytosolic compartment because blockage of membrane anion channels, the only known pathway for the passive diffusion of $O_2^{\cdot-}$ across the membranes, has no influence on measurement outside the cells. Furthermore, the absence of an inhibitory effect of the NADPH oxidase inhibitor, DPI, suggests that extracellular $O_2^{\cdot-}$ does not arise primarily from membrane-associated oxidoreductases that have been identified in endothelial and other cells (153,229). Finally, inhibition of electron transport, both before and after the Q cycle, results in paradoxical elevations in extracellular $O_2^{\cdot-}$ release. This observation suggests that the yet unidentified source of extracellular $O_2^{\cdot-}$ might be sensitive to conditions of disordered metabolism or “chemical hypoxia”, and that simply attempting to block mitochondrial electron transport in whole tissues or cells in order to determine the potential role of mitochondria as a source of ROS can lead to potential errors in interpretation.

3.5.1 Influences of electron transport on extracellular $O_2^{\cdot-}$ formation

Our previous studies have demonstrated that increased cyt c reductions caused by heat-stressed skeletal muscle can be almost entirely blocked by SOD (247), suggesting that other reducing equivalents such as $^\circ$NO (12), or oxidizing agents such as $H_2O_2$ (232), are
not important components of the signal measured by cyt c. We have also shown that an “intracellular” $O_2^{•−}$ signal produced during heat stress (using a fluorescent probe for $O_2^{•−}$) has a similar time course to that of extracellular $O_2^{•−}$ (247), suggesting the signals arise from the same source. Of the known intracellular sources of ROS in cells, mitochondria are well recognized and seemed to be the most likely candidates because mitochondrial oxygen consumption and ATPase activity are increased in skeletal muscle during heat exposure (28). Furthermore, increases in temperature can markedly influence membrane fluidity, which is known to be of critical importance for mitochondrial formation of $O_2^{•−}$ by ubiquinone at the inner mitochondrial membrane (82). Assuming mitochondrial $O_2^{•−}$ could exit the mitochondria in the intact cell (230), these conditions would improve the probability of electron leakage, promoting reduction of $O_2$ to $O_2^{•−}$ (222). Therefore, our initial working hypothesis was that mitochondria were the source of both intracellular and extracellular $O_2^{•−}$ formation in heat stress, and we thus embarked on blocking mitochondria at relevant sites of electron transport.

The paradoxical results were unexpected and may reflect, in part, experimental constraints of mitochondrial blockade in intact tissues. In all cases, we chose to use the highest concentrations that were available in the literature for whole tissue experiments because of concerns regarding diffusion distances and sufficient intracellular concentrations. Usually, these comprised the upper limit of solubility that could be attained in buffer solutions, without reaching potentially damaging solvent concentrations (i.e. < 100 mM DMSO). We quickly encountered the limitations of this approach. First, by comparison with isolated mitochondrial experiments, there is no control of substrate
utilization or availability in intact tissues. Effective blockade of NADH as a substrate through complex I can potentially be overcome in the intact cells by their ability to provide reducing equivalents to complex II through succinate or, for example, by the oxidation of fatty acyl coenzyme A through the flavin-linked electron transferring flavoprotein at the level of ubiquinone. Second, there is no control of \([ADP]\) and therefore the measured metabolic rate, as seen in Fig. 3.3, may be variable and is a reflection of resting ATPase activity rather than actual mitochondrial function. Metabolic demand can vary depending on the state of the cell, (i.e. membrane potential, \(Ca^{2+}\) leakage, myofibrillar tone, etc.) and is no doubt altered to some extent by the stress of mitochondrial poisons. Therefore, there is no standardization of ATP turnover or \(O_2\) consumption to compare the effect of mitochondrial blockade. Third, at any given time, resting \(O_2\) consumption may comprise a small proportion of the tissue’s total potential to consume \(O_2\) (i.e. far away from Vmax for cytochrome oxidase) as skeletal muscle can increase its metabolic rate by well over an order of magnitude as ADP and other regulators increase. This means that if a given inhibitor has blocked 90% of all potential mitochondrial electron transport, sufficient pathways of electron transport may still be available to sustain overall ATP and creatine phosphate concentrations at near normal levels. These limitations do not exist when studying isolated mitochondria, where substrates and [ADP] are controlled and where electron transport blockers become most effective tools. Some components of the problem are illustrated in Fig. 3.3, in which the highest concentration of rotenone we could provide to the tissue blocked only 25% of resting \(O_2\) consumption and rotenone + TTFA blocked only 55%. We can conclude that we were unable to load sufficient concentrations of rotenone to the mitochondria or that
the mitochondria of skeletal muscle, in their intact intracellular environment, are extremely flexible in their ability to effectively shuttle alternate sources of electrons, thus bypassing our specific manipulations of electron transport.

Nevertheless, the data are insightful and seem to point to a more general and obvious conclusion. In virtually every attempt to block mitochondrial electron transport, (with the exception of complex I inhibition by rotenone alone, which only inhibited respiration by 25%), there was a paradoxical and marked stimulation of extracellular $O_2^{•−}$ formation, both at rest and during heat stress. Based on the early work of Boveris et al. (22), we might have expected that complex III inhibition (antimycin A) would increase $O_2^{•−}$ formation because of its effect on increasing the electrical potential at the Q site. Though blocking complex I alone by rotenone (185,202) or amobarbital (5) has usually been shown to decrease ROS formation, on occasion complex I inhibition has been shown to increase ROS, in certain experimental conditions (227). Therefore, it is possible that in our experiments, these two opposite effects on ROS formation by rotenone blockage may counteract each other, resulting in a non-significant effect as observed in Fig. 3.2. However, we did not expect that by combining the blockade of complex I and II together (rotenone + TTFA), there would be an almost identical effect on $O_2^{•−}$ formation when compared to blocking complex III, as this treatment should unload the electron potential at the Q cycle. Thus, it seems that by poisoning electron transport by multiple pathways we have either unveiled a new extracellular $O_2^{•−}$ generator that is sensitive to the metabolic state of the cell, or somehow stimulated the existing extracellular generators that are active in the tissue at rest or in heat stress. By comparing the magnitudes of the
changes in the top and bottom panels of Fig. 3.2, it can be seen that the net elevation in cyt c reduction in response to mitochondrial blockade was not substantially altered between heat stress and resting conditions, even though metabolic rate was predictably increased at the higher temperature. This suggests that heat stress does not further amplify the influence of metabolic inhibition or vice versa. Thus, we speculate that metabolic stress induced by “chemical hypoxia” and heat stress may operate by separate mechanisms or at least operate at non-interacting control points of a single mechanism.

In summary, coupled with the results for anion channel blockade, we see no evidence that the extracellular O$_2^{-}$ generator at rest or during heat stress could arise from the mitochondria.

What could be responsible for the influence of metabolic blockade on extracellular ROS formation? One potential source in the intact cell could be the xanthine oxidase system. A build-up of purine nucleotide catabolic products such as hypoxanthine can provide substrate for xanthine oxidase, producing O$_2^{-}$ and H$_2$O$_2$ (84). Other sources could involve release or relocalization of Ca$^{2+}$ from mitochondria or other organelle stores in response to metabolic blockade, possibly activating Ca$^{2+}$-dependent ROS generating systems such as the phospholipase A$_2$ (PLA$_2$) pathway (157). An increase in free Ca$^{2+}$ could also stimulate proteolytic conversion of xanthine oxidase from xanthine dehydrogenase to produce O$_2^{-}$ (85).

### 3.5.2 Blockage of membrane-associated NADPH oxidases

Many studies have shown that membrane-associated NADPH oxidases provide important
sources of ROS in a variety of normal and abnormal conditions (63,133,229). DPI and its analogs are most commonly used as potent inhibitors of these oxidases (79) via the iodonium modification of heme b at the flavocytochrome b (63). Since extracellular $O_2^{•−}$ did not appear to diffuse across membrane anion channels, a reasonable alternative hypothesis was that a membrane NADPH oxidase might be responsible. Such oxidases do not require channels for $O_2^{•−}$ release and are capable of moving an electron directly across the cell membrane to reduce $O_2$ at least in inflammatory cells (197). Of particular importance for this study is the potential existence of NADPH oxidases in endothelial cells (224) because the diaphragm contains a large capillary endothelium. Whether they exist or play a role in skeletal muscle membranes is not well studied. Our results show that general inhibition of NADPH oxidases with DPI did not inhibit, but rather stimulated $O_2^{•−}$ release at both 37°C and 42°C, much like mitochondrial blockade. Therefore, the results are not consistent with membrane NADPH oxidases being the primary source of $O_2^{•−}$ release in a resting control state or in heat stress. Since DPI analogs in sufficient concentrations can also block complex I of the mitochondria (133), we speculate that the same phenomenon observed for mitochondrial blockade could occur with DPI administration. That is, metabolic inhibition by DPI unmasked the same source of extracellular $O_2^{•−}$ formation shown previously with metabolic disturbances induced by other mitochondrial blockers. It is still possible that an underlying NADPH oxidase mechanism is acting in some conditions in this preparation, but it is unlikely to be the only or even the primary source in this model. The results reemphasize the complexity of attempting to block specific pathways of ROS formation in the intact tissue, as there
could be secondary influences on ROS formation by alternative pathways. This point is largely overlooked in studies of this nature.

3.5.3 Membrane anion channel blockage

Both probenecid (35) and DIDS (214) have been used extensively as anion channel blockers. The most conclusive results of the study are the lack of effects of high concentrations of these agents on the measured variables. This all but rules out known mechanisms of passive diffusion as a mechanism for extracellular $O_2^{•−}$ release from intracellular sources in this model. The role of anion channels in $O_2^{•−}$ release have been studied extensively in isolated red blood cells (139) and are also believed to provide a pathway for $O_2^{•−}$ release from mitochondria in cardiomyocytes (230). However, our data suggest that there must be an alternate electron flux system, which works either by a yet unidentified anion channel or by directly conducting electrons across cell membranes (197).

3.5.4 Conclusions and speculations

The results suggest that extracellular $O_2^{•−}$ release may not simply reflect a spillover of reduced oxygen from intracellular sources such as mitochondria, since the only mechanisms known for $O_2^{•−}$ transport across either cell or mitochondrial membranes (i.e. anion channels) do not appear to be involved in this preparation. If the molecular sources of intra- and extracellular $O_2^{•−}$ are the same, then a specific membrane-associated mechanism is likely that would require access to both intra- and extracellular compartments. Pathways that could act in this way might include the xanthine oxidase
(208), nitric oxide synthase (241) or PLA₂-related degradation pathways such as cyclooxygenase and lipoxygenase (124,157), and further studies are required.

The stimulating effects on $\text{O}_2^{•−}$ release by the variety of blockers used in this study, (usually used to demonstrate the inhibition of ROS formation) appear puzzling but reveal that the mechanisms for ROS production in the intact cell or tissue are very complex and are probably much more interactive than is often appreciated. In a general way, mitochondrial blockage or possibly DPI administration can increase the reducing potential of the cell, thus elevating the probability for the intracellular reduction of $\text{O}_2$ to $\text{O}_2^{•−}$. How this occurs, we can only speculate. Some $\text{O}_2^{•−}$ generating systems such as xanthine oxidases, lipoxygenases or cyclooxygenases, require NAD(P)H. Since these are elevated in chemical hypoxia, they could influence the rate of a variety of redox reactions (124). Alternatively, other transition metals in the cell could be made more susceptible to participating in single electron transfer reactions to $\text{O}_2$ in a highly reducing environment. It is also possible in the intact living cell that regulation of redox tone is an ongoing process with alternative back-up mechanisms. Thus, when one generator responsible for pushing forward the intracellular or extracellular oxidizing environment is inhibited, another may become up-regulated to maintain local $\text{O}_2^{•−}$, peroxide or thiol “tone.” This intriguing idea, which has been the subject of a recent review (196), would suggest that intracellular redox sensors are actively engaged in a normal regulatory pathway through the possible orchestration of multiple ROS-generating sites. Such a possibility makes finding unique sources of ROS formation in a specific biological condition, such as heat stress, extremely challenging in intact tissues.
Table 3.1. Test of collision probability between cyt c and ROS. N = 6 in each group from 3 rats. * P < 0.05, significantly different from controls (5 μM).

<table>
<thead>
<tr>
<th>Min</th>
<th>% (4 to 5 μM cyt c)</th>
<th>% (3 to 5 μM cyt c)</th>
</tr>
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<tbody>
<tr>
<td>15</td>
<td>93 ± 9 (n.s.)</td>
<td>74 ± 14</td>
</tr>
<tr>
<td>30</td>
<td>92 ± 8 (n.s.)</td>
<td>69 ± 10*</td>
</tr>
<tr>
<td>45</td>
<td>92 ± 10 (n.s.)</td>
<td>75 ± 10*</td>
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Fig. 3.1. Complex III inhibition with antimycin A. Results expressed as absolute cyt c reduction. Antimycin A significantly increased cyt c reduction at 37°C and 42°C (N=6). * P<0.05 between drug treatments and controls at 42°C; † P<0.05 between drug treatments and controls at 37°C.
Fig. 3.2. Summarized data showing net differences from matched controls in response to blockers of mitochondria and NADPH oxidases at 37°C and 42°C. * P<0.05 (ANOVA contrasts) between drug treatment and corresponding non-drug treatment at the given temperature.
Fig. 3.3. Inhibition of oxygen consumption with rotenone alone and rotenone + TTFA in intact tissues. * P<0.05, between drug-treated tissues and non-drug treated tissues (N=6 from 5 rats). † P<0.05, between rotenone alone and rotenone + TTFA (N=6 from 5 rats). ‡ N=5 for this group due to the removal of one data point determined by an outlier test.
Fig. 3.4. Summarized data showing net differences from matched controls in response to anion channel blockers at 37°C and 42°C. Neither probenecid nor DIDS had any significant effect on cyt c reduction at both 37°C and 42°C (N=6). At 42°C, a slight trend of increased inhibition due to DIDS can be observed. However, as a whole, the DIDS-treated data did not show any significant inhibition on O$_2^*$ release from the tissue at 42°C (N=6). † P=0.36; ‡ P=0.19 between drug treatment and corresponding non-drug treatment at the given temperature.
CHAPTER 4

THE SUPEROXIDE RELEASE IN SKELETAL MUSCLE IS DEPENDENT ON ARACHIDONIC ACID METABOLISM THROUGH LIPOXGENASE

4.1 Abstract

Previous studies have shown that superoxide (O$_2^•$-) release from diaphragm is not dependent on mitochondrial electron transport, NADPH oxidase activity or the integrity of membrane anion channels. This study hypothesized that O$_2^•$- release is linked to metabolism of arachidonic acid, specifically via phospholipase A$_2$, cyclooxygenase, lipoxigenase and cytochrome P450-dependent monooxygenase pathways. Phospholipase A$_2$ inhibition with manoalide significantly decreased O$_2^•$- release. In downstream pathways of arachidonic acid metabolism, neither the inhibition of cyclooxygenase with indomethacin nor inhibition of cytochrome P450-dependent monooxygenase with SKF 525A blocked O$_2^•$- release. However, lipoxigenase inhibition with 5,8,11,14-eicosatetraynoic acid caused a marked reduction of O$_2^•$- release. Recent research has also shown that arachidonic acid release is linked to nitric oxide synthase-
dependent peroxynitrite formation. Nitric oxide synthase inhibition resulted in a significant decrease of $O_2^{-}$ release to approximately the same extent as phospholipase A$_2$ inhibition, suggesting the likelihood of this interaction. Confocal studies demonstrated that lipoxygenase inhibition had no significant influence on intracellular $O_2^{-}$ formation, indicating that intra- and extracellular sources of $O_2^{-}$ must arise from different mechanisms. These data show for the first time that extracellular $O_2^{-}$ release, is dependent on arachidonic acid metabolism specifically through lipoxygense activity.

4.2 Introduction

Superoxide ($O_2^{-}$) is formed extracellularly by many cell types, most notably inflammatory cells, where its formation plays a critical role in host defense. In other cell types, the function of extracellular $O_2^{-}$ is less well understood. However, like nitric oxide ($\cdot$NO), it may play a role in regulation of local vascular smooth muscle (158), regulating the redox tone of the extracellular matrix (244), or affecting local $\cdot$NO bioavailability (167). The discovery of extracellular superoxide dismutase (73) is another indication that extracellular $O_2^{-}$ formation may be biologically relevant and is probably under local regulation.

A number of studies have demonstrated that isolated (181) or perfused (117) skeletal muscle produces considerable extracellular $O_2^{-}$. Much like the formation of extracellular $\cdot$NO (111), the level of this radical increases with increased contractile activity (117,181). In addition, recent studies from our laboratory have documented a
significant rise in O$_2^•^-$ release in skeletal muscle (diaphragm) under conditions of mild heat exposure (247), another stimulus associated with exercise.

The organelle or molecular source of O$_2^•^-$ release in muscle is not known. We previously demonstrated that it does not appear to be attenuated by inhibition of electron transport, inhibition of NADPH oxidases or by blockage of membrane anion channels (249-251). Therefore, it appears likely that the O$_2^•^-$ generator is associated with the extracellular membrane and that it can pass electrons or O$_2^•^-$ directly across the membrane by an unknown mechanism. Of the possible sources, nitric oxide synthase (NOS) and enzymes associated with arachidonic acid (AA) metabolism appear to be good candidates, as they are both present in or near the plasma membrane in muscle. Potential interactions of these pathways are illustrated in Fig. 4.1.

The following hypotheses were tested. H$_1$: O$_2^•^-$ release at rest and in heat exposure is related to phospholipase A$_2$ (PLA$_2$) activity, one of the primary upstream components of AA metabolism. This would be in agreement with studies that have associated O$_2^•^-$ formation during muscle contraction with PLA$_2$ activity (157). H$_2$: Downstream pathways of extracellular O$_2^•^-$ release are related to the activities of cyclooxygenase (COX), cytochrome P450-dependent monooxygenase (P450) and/or lipoxygenase (LOX). H$_3$: Increased O$_2^•^-$ release depends on stimulation of NOS activity. H$_4$: The major source for O$_2^•^-$ release is independent of the source of intracellular O$_2^•^-$ production. All these hypotheses were tested based on corresponding blockers as shown
in Fig. 4.1. The results demonstrate for the first time the role of LOX as a critical downstream pathway of extracellular $\text{O}_2^-$ generation in isolated diaphragm tissue. Furthermore, evidence is presented that NOS and PLA$_2$ play important roles in upstream regulation of $\text{O}_2^-$ generation.

### 4.3 Methods

#### 4.3.1 Surgical procedures

Male Sprague-Dawley rats (350–450 g) and Swiss-Webster mice (25–35 g) were housed and treated according to approved protocols of The Ohio State University Institutional Laboratory Animal Care and Use Committee. The procedures have been described previously (247). In brief, rats were anesthetized with sodium pentobarbital (~40 mg/kg) or ketamine (~76 mg/kg) and xylazine (~15 mg/kg), tracheotomized and ventilated. Their blood was heparinized (~500 U/kg) and the diaphragms were retrograde perfused from the inferior vena cava with oxygenated Ringer’s solution. This was performed to lower leukocytes and hemoglobin that may affect the cytochrome c (cyt c). Each diaphragm was removed and dissected into several muscle strips (~0.4-0.7 cm wide) attached to corresponding central tendon and rib. The strips were kept in Ringer’s solution (in meq/L: 21 NaHCO$_3$, 1.0 MgCl$_2$, 1.2 Na$_2$HPO$_4$, 0.9 Na$_2$SO$_4$, 2.0 CaCl$_2$, 5.9 KCl, 121 NaCl, 2.07g/L glucose and 10 $\mu$M d-tubocurarine) on ice, and bubbled with 95% O$_2$ and 5% CO$_2$, prior to use.
4.3.2 The cytochrome c assay

Similar to previous descriptions (247), extracellular O$_2$•$^-$ release was measured with the cyt c assay in a darkened room. Cyt c is highly sensitive to O$_2$•$^-$ and can be easily reduced through one-electron donation from O$_2$•$^-$. To minimize the possible non-cyt c-specific interference, spectral measurements of cyt c reduction were calculated by taking the difference between the peak absorbance at 550 nm and the average of the values at 540 nm and 560 nm (117,247). The extinction coefficient used for cyt c was 18.5 x 10$^3$ M$^{-1}$cm$^{-1}$ (117,143,247).

4.3.3 Treatments

The diaphragm strips were incubated with or without blockers for 30 min, on ice. Each muscle strip was then loaded with ~2 g preload tension to attain approximate optimum length, and equilibrated at 37°C in a 3.0 ml water-jacketed minibath (Radnoti, Monrovia, CA) with or without fresh blocking solution for 10 min. Control strips were exposed to 37°C or 42°C for 45 min in 5 µM cyt c solution, without blockers. Treated strips were handled identically except the corresponding blockers were added. The reduction of cyt c in each minibath was monitored by quickly transferring 1 ml of the bath solution to a cuvette in a diode-array ultraviolet-visible spectrophotometer (HP 8452A, Hewlett-Packard) and recording the full spectra. Then the cuvette solution was immediately transferred back to the tissue bath.

The following blockers were used: manosalide (Kamiya Biomedical CO., Seattle, WA), indomethacin, SKF 525A (proadifen) and 5,8,11,14-eicosatetraynoic acid (ETYA) (all
from Sigma), N\textsuperscript{G}-Nitro-L-arginine-methyl ester (L-NAME) and its non-reactive isomer N\textsuperscript{G}-Nitro-D-arginine-methyl ester (D-NAME, both from Alexis Biochemicals, San Diego, CA). The stock solutions of manoalide, indomethacin and ETYA were made with dimethyl sulfoxide (DMSO, Sigma), and all others were made with double distilled water. For experiments requiring DMSO, the same amount of DMSO was added to control tissues. DMSO concentrations were kept below 0.5% v/v to reduce possible effects on the muscle (184). Each blocker was pre-tested using cyt c assay in the absence of tissues, to check its reactivity with cyt c and when present, appropriate corrections were made in the final analysis. The concentrations of all blockers were kept at high levels, based on literature values, because of the necessity for diffusion into whole tissues. All treatments had a relatively low chemical effect on cyt c reduction, i.e. less than ~18% of cyt c fully reduced by ascorbic acid. We found that this degree of effect did not cause artifacts related to the probability of collision between cyt c and reactive oxygen species (ROS) (see table 3.1, chapter 3). When necessary, the pH of the buffer was re-adjusted to 7.4-7.6 prior to all experiments. All final data were corrected for the direct effects between cyt c and the specific blockers. SKF 525A did not dissolve well in Ringer’s solution at 50 \textmu M, due to precipitate formation with NaHCO\textsubscript{3} and Na\textsubscript{2}HPO\textsubscript{4}. Thus, for this experiment, the buffer solution was prepared without these two components, and the pH of oxygenated buffer was titrated to 7.4-7.6 by addition of NaOH.
4.3.4 Laser scan confocal microscopy

Confocal experiments were designed to identify whether the most effective blocker (ETYA) also inhibited intracellular ROS formation. The experiments were performed as previously described (247). Hydroethidine (HE) was used for the detection of intracellular ROS and is particularly sensitive to $\text{O}_2^{\cdot*}$, hydroxyl radical ($^\bullet\text{OH}$) and peroxynitrite ($\text{ONOO}^-$), but not to hydrogen peroxide ($\text{H}_2\text{O}_2$) (3,247). HE stocks were prepared in $N,N$-dimethylacetamide (Acros Organics). In the presence of ROS, HE becomes oxidized to ethidium (ET), which was used as the indicator of intracellular ROS production (3,31,157,247). Our previous studies (247) have shown better HE loading and improved fluorescent detection in mouse diaphragms when compared to rat under the same conditions, therefore mouse diaphragms were used for confocal experiments. Mice were anesthetized with the combination of ketamine (~166 mg/kg) and xylazine (~33 mg/kg), and diaphragms were quickly removed. Hemidiaphragms were loaded in a solution of 88 µM HE and 4 mM 2,3-butanedione monoxime (BDM, an actin-myosin complex blocker used to reduce movement artifact, Acros Organics), with or without 100 µM ETYA, in oxygenated Ringer’s solution, in the dark, on ice for 1 hr. After loading, muscles were washed for 10 min with Ringer’s solution and stretched to approximately 120% of relaxed length on the stage of a laser scan confocal microscope (Zeiss 410, Carl Zeiss, Germany). After a 30 min equilibration at 37°C with a constant superfusion, both treated and untreated groups were exposed to 35 min of 42°C. Measurements of ET fluorescence were taken every 5 min. The setup and analysis for laser scan microscopy has been described in detail previously (247).
4.3.5 Test of the in vitro antioxidant effects of ETYA (100 μM) on $O_2^{•−}$

Since LOX inhibitors can act as antioxidants, it is necessary to test the $O_2^{•−}$ scavenging ability of ETYA. The reaction medium included: cyt c (10 μM), catalase (2000 U/ml, Sigma), xanthine (0.25 mM, Sigma), ethylenediaminetetraacetic acid (EDTA, 2 mM, Acros Organics) and PBS, pH=7.55. Xanthine oxidase (0.75 U/ml, Sigma) was added to begin the generation of $O_2^{•−}$. Our results demonstrated that 100 μM ETYA had no appreciable scavenging effect on the $O_2^{•−}$ (data not shown).

4.3.6 Statistical and graphical analysis

Data were analyzed using a multi-way ANOVA and expressed as means ± SE (JMP, SAS Institute, Cary, NC). The rat or mouse was treated as a random variable with drug-treatment and time being factors of interest. The differences in mean values between treatment and non-treatment at each specific time point were determined by post ANOVA contrasts. $P < 0.05$ was considered to be statistically significant.

4.4 Results

As shown in Fig. 4.2, after 30 min of PLA$_2$ inhibition with manoalide (10 μM), there was a significant inhibition of the rate of cyt c reduction, at both 37°C (N=6, P<0.02) and 42°C (N=8, P<0.02). Fig. 4.2 is used to illustrate a complete picture of the typical responses of these kinds of experiments over time. From such data, the net changes in cyt c reduction from matched controls were calculated and used to summarize the results (Fig. 4.3).
As shown in Fig. 4.3, indomethacin (500 μM), a blocker of the COX pathway, had no significant inhibitory effect on cyt c reduction at 37°C or 42°C (N=6). Interestingly, and contrary to our expectations, it significantly increased cyt c reduction at 37°C (N=6, P<0.01). Similarly, inhibition of the P450 pathway with SKF 525A (50 μM) did not diminish cyt c reduction and promoted reduction at both 37°C and 42°C after 30 min (N=5, P<0.01). However, LOX inhibition (100 μM ETYA) had a striking inhibitory effect on the rate of cyt c reduction at both 37°C (N=8, P<0.01) and 42°C (N=6, P<0.01), particularly at time points of 30 and 45 min. Since our in vitro experiments (Methods Section) demonstrated that ETYA has no O$_2^•$ scavenging ability at these concentrations, we conclude that the primary pathway of extracellular O$_2^•$ formation is either directly or indirectly through the action of LOX. The stimulatory effect of blockage of the alternative pathways of AA metabolism (i.e. COX and P450 inhibition) suggests the possibility that these blocking experiments shuttled AA substrates through the LOX pathway.

Since NOS is capable of producing O$_2^•$ independently under some conditions of L-arginine depletion (241), and because ONOO$^-$ can act as a stimulator of AA metabolism through phospholipase activation (87), we tested its potential role in promoting extracellular O$_2^•$ formation. Independent experiments eliminated *NO’s potential role in directly reducing cyt c. Fig. 4.4 illustrates that after 15 min at 42°C and 30 min at 37°C, NOS inhibition with L-NAME (10 mM) caused a significant attenuation of cyt c.
reduction (N=8, P<0.05). However, D-NAME (10 mM) had no inhibitory effect on cyt c reduction at either 37°C or 42°C (N=4). This suggests that NOS also plays a direct or indirect role in the formation of extracellular $O_2^{\cdot-}$.

We also tested whether ETYA inhibited intracellular ROS production. Despite its striking effects on extracellular $O_2^{\cdot-}$ release, Fig. 4.5 shows that it has no significant effect on intracellular ROS formation during heat stress in the mouse (N=5). This suggests that although the time courses of intra- and extracellular $O_2^{\cdot-}$ formation appear similar, as shown in our previous work (247), they seem to be generated by independent sources or by different molecular mechanisms.

4.5 Discussion

The results suggest that the primary source of extracellular $O_2^{\cdot-}$ is a reflection of downstream pathways of AA metabolism and specifically, the activity of LOX. Blockage of this pathway caused near complete inhibition of $O_2^{\cdot-}$ release, both at rest and during exposure to mild heat. Furthermore, blockage of alternative paths of AA metabolism (COX and P450) stimulated production, which is consistent with competition for AA substrates between these enzyme systems. The confocal experiments suggest that the pathway of extracellular $O_2^{\cdot-}$ formation is separate from the intracellular source and is consistent with previous data that demonstrated that anion pores, known as the major mechanisms for intracellular derived $O_2^{\cdot-}$ across cell membranes (139), do not play a role in this setting (251).
4.5.1 The measurement of $O_2^{•−}$ release from diaphragm

Superoxide dismutase-inhibitable cyt c reduction is one of the few methods that give reliable measurements at low concentrations of $O_2^{•−}$ in the extracellular environment. Other methods, often used for inflammatory cell studies, such as spin trapping, can reflect both intra- and extracellular $O_2^{•−}$ formation. Furthermore, the paramagnetic radical adducts formed during spin trapping are quickly reduced to non-detectable diamagnetic products when studying whole tissue (15). Since our previous experiments demonstrated that the $O_2^{•−}$ signal observed during heat stress could be completely inhibited by addition of superoxide dismutase to the bath (247), we are confident that the primary signal arising from the muscle during heat stress is $O_2^{•−}$.

Although nitric oxide (•NO) has the ability to reduce cyt c, this is not problematic when appropriate precautions are used. First, •NO initially forms a nitrosyl-ferric complex that exhibits a strong absorption peak at 563 nm. This is followed by a further reduction peak at 550 nm in a later phase of the reaction (after 1-4 hr) (12). In our system, this characteristic peak “shift” over the time of data collection was never observed. Furthermore, using the method of subtracting the average of 540 nm and 560 nm from the peak at 550 nm, would, if anything, result in a loss of apparent absorbance at 550 nm after reaction of •NO with cyt c, which was also not observed in these experiments.
4.5.2 Inhibition of AA metabolism and the potential contribution of NOS

Previous research has shown that ROS formation can be directly or indirectly related to the activity of PLA2 in skeletal muscle (157,239,240), although the mechanism is not well understood. Exposure of living cells to AA alone can result in the massive formation of ROS (240), suggesting that the pathway may be downstream from PLA2. Manoalide is an effective blocker of PLA2 and works by irreversible modification of lysine residues on PLA2 (16). Our data (Fig. 4.2 and 4.3) have shown that ROS release is greatly reduced after PLA2 blockage, but it is not completely inhibited. It is possible that other phospholipases, not inhibited by manoalide, may have contributed substrates for downstream AA metabolism and consequent O$_2^{•−}$ formation. Alternatively, some enzymes such as 12/15-lipoxygenase can act directly on membrane phospholipids, thus bypassing PLA2 (215).

NOS inhibition with L-NAME also inhibited cyt c reduction, both at rest and during heat exposure. The magnitude of inhibition was approximately equal to that of PLA2 treatment. D-NAME had no such effect, suggesting this is a specific effect of NOS inhibition. As mentioned earlier, it is unlikely that *NO directly reduced cyt c in this study to any significant extent. However, there are at least three conceivable mechanisms by which NOS could influence extracellular O$_2^{•−}$ formation. First, NOS is capable of producing O$_2^{•−}$ directly (174,241). The physiologic relevance of this reaction is not well known, but we speculate that it may play a very minor role in our model as it is believed to require conditions of low L-arginine availability (241), which is unlikely using freshly excised muscle. A second factor to consider is that the *NO generated by NOS could
react directly with $O_2^{•−}$ to form ONOO$^−$. If this were an important phenomenon, NOS inhibition would have increased the $O_2^{•−}$ available for the cyt c reaction. However, it had the opposite effect. A third mechanism, involves the potential role of ONOO$^−$ in upregulating PLA$_2$ activity (87). Our data strongly suggest that the latter mechanism may be most important (Fig. 4.4).

### 4.5.3 The LOX pathway as a potential $O_2^{•−}$ generator

Of the three major downstream pathways of AA metabolism, only LOX inhibition significantly blocked cyt c reduction. Blockage of the alternative pathways, increased cyt c reduction. Interestingly, both COX (164,236) and P450 (20,170) are well known as ROS generators in other systems and LOX is not generally viewed as a major contributor to ROS since it has not been studied extensively. The fact that blockage of both COX and P450 augmented cyt c reduction also provides circumstantial evidence for a role of LOX, since competition for AA substrates between these alternative pathways has been observed in studies on other systems (121,146).

How does LOX create $O_2^{•−}$? Though less well recognized, LOX has been shown to be either directly or indirectly related to ROS formation (124,127,138,239,240), but the mechanism is not fully understood. There are at least two possible processes. First, non-heme iron in LOX can cycle between ferrous ($Fe^{2+}$, inactive) and ferric ($Fe^{3+}$, active) forms during the catalysis, resulting in the formation of lipid peroxy radical intermediates by the addition of oxygen molecules to AA substrates. During this process, it is possible $Fe^{2+}$ could directly transfer one electron to molecular oxygen to form $O_2^{•−}$ (24). Second,
the products of LOX catalysis such as leukotriene B₄ may play an autocrine or paracrine role in stimulating ROS formation by secondary mechanisms (239,240).

The absence of an effect of anion channel blockers on extracellular \( \text{O}_2^{\bullet-} \) release in this model, as shown in a previous study (251), suggests that the mechanism is likely to involve a molecular system bound to the cell membrane that, by its location, overcomes the hydrophobic barrier for movement of a charged molecule to the extracellular environment. Membrane-bound LOX appears to fit this role very well and could conceivably produce \( \text{O}_2^{\bullet-} \) without the need of specific channels or other more complicated mechanisms. It is also possible that high environmental \( \text{O}_2 \), required of isolated tissue experiments, could amplify the \( \text{O}_2^{\bullet-} \) signal seen via this pathway.

There are other related issues with regard to downstream blockage of AA metabolic pathways: 1) Although indomethacin is a well-known, and powerful inhibitor for COX, it can also have an inhibitory effect on LOX (236). Our data from COX and LOX blockage (Fig. 4.3) strongly suggest, however, that indomethacin had either no significant or a very weak inhibitory effect on LOX, even though a high concentration of this drug was used; 2) Many LOX inhibitors have antioxidant properties. For example, we were unable to use another LOX inhibitor, nordihydroguaiaretic acid (NDGA) (220) because we found it to be a very strong reducing agent of cyt c, and this greatly interfered with the cyt c spectra. Furthermore, NDGA is well known as an antioxidant. We found ETYA had no appreciable antioxidant effect on \( \text{O}_2^{\bullet-} \) released from the tissue, which is consistent with a former study (220) which demonstrated that ~ 30 fold higher concentrations, than that
necessary to inhibit LOX, are required to show significant antioxidant activity. Furthermore, though ETYA had a small reducing effect on cyt c, it could be easily controlled for in this study; 3) Although ETYA may have some inhibitory effect on COX (68), it seems unlikely to play a major role, since a more effective drug for COX inhibition (indomethacin) had no inhibitory effect on $O_2^{•-}$ release (Fig. 4.3).

### 4.5.4 Summary and implications

The results suggest that the AA-LOX pathway is one of the major sources for extracellular $O_2^{•-}$ release in this model. Although not tested under other physiologic stimuli, such as muscle contraction (117,181,182), the fact that LOX inhibition was effective during resting conditions suggests that it may be of more general relevance. Our results are also consistent with Woo et al. (239), who demonstrated that ROS are generated, not through the COX pathway, but through the cytosolic PLA$_2$-LOX pathway in the study of rat-2 fibroblasts stimulated by tumor necrosis factor-$\alpha$. It is interesting to speculate that extracellular $O_2^{•-}$ plays some important, but as yet unidentified role, such as altering the extracellular bioavailability of $\bullet$NO. The fact that ONOO$^-$ has the potential of providing a positive feedback signal on PLA$_2$ activity, thus driving this response, may have important implications for other conditions of stress, such as endotoxin-induced sepsis (37). Recently, Hall et al. (89) have demonstrated that in whole body heat stress, there is a significant and potent elevation in oxidative stress and $\bullet$NO formation, which contributes to its pathology. It is possible that AA metabolism may be a contributory pathway to the pathology of heat stress in some tissues. In skeletal muscle, elevations in temperature may be considered a more “normal phenomenon” as
they are natural heat generators and during exhaustive exercise, muscle temperatures can exceed 43°C (28). Therefore, the role of such mechanisms in skeletal muscles may also be uniquely different from other tissues.
Fig. 4.1. Schematic representing agents used to inhibit the enzymes involved in the AA cascade via NOS, PLA₂, COX, LOX and P450.
Fig. 4.2. PLA₂ inhibition with manoalide. After 30 min, manoalide significantly decreased cyt c reduction at both 37°C (N=6) and 42°C (N=8). * P<0.02 between drug treatments and controls at 42°C; † P<0.02 between drug treatments and controls at 37°C.
Fig. 4.3. Summarized data showing net differences from matched controls in response to various blockers in the AA-LOX pathway at the given temperature. * P < 0.05, significant difference between drug treatment and corresponding non-drug-treatment.
Fig. 4.4. Summarized data showing net differences from controls in response to L-NAME and D-NAME at 42°C and 37°C. * P<0.05 between drug treatment and corresponding non-drug-treatment at the same temperature.
Fig. 4.5. Confocal study: the effect of LOX inhibitor ETYA on intracellular $O_2^{•−}$ formation in heat stress. There is no significant effect of ETYA on $O_2^{•−}$ formation (N=5).
5.1 Abstract

Hypoxia/reoxygenation is believed to produce reactive oxygen species (ROS) during reoxygenation. However, whether ROS are directly generated during hypoxia in skeletal muscle remains controversial. Isolated rat diaphragm strips were exposed to alternating normoxia (95% O₂) or hypoxia (0-21% O₂) superfusates. The conversion of dihydrofluorescein (Hfluor) to fluorescein (Fluor) was used for the determination of cellular hydrogen peroxide formation. In other tissues, the conversion of hydroethidine (HE) to ethidium (ET) was used as an indicator of superoxide formation. Tissue fluorescence was monitored using either reflected or transmitted fluorescence. Autofluorescence measurements in unloaded tissues decreased in the FAD range during hypoxia, which overlapped both the ET and Fluor fluorescence signals. Despite this, in tissues loaded with HE or Hfluor probes, there was a marked increase in fluorescence with hypoxia and a rapid decrease with reoxygenation. Secondary tests were performed by loading muscles with the oxidized forms of the probes. Similar patterns of fluorescent change were observed for both oxidized probes, suggesting that the apparent hypoxia
signal includes artifacts that are not specific for ROS. Therefore, corresponding antioxidant experiments were performed. Ebselen, a glutathione peroxidase mimic functioning as a H₂O₂ scavenger, completely diminished the hypoxia-induced signal. However, there was no consistent effect of the superoxide scavenger, Tiron, on the hypoxic signal using HE loaded tissues, suggesting a lack of detectable levels of superoxide during hypoxia. These results suggest that critical levels of low environmental O₂ appear to induce hydrogen peroxide formation in isolated skeletal muscle. However, the reactive oxygen signal is a complex superposition of additional signals arising from autofluorescence and additional artifacts associated with hypoxic exposure.

5.2 Introduction

The formation of reactive oxygen species (ROS) in skeletal muscle has been described in conditions of heat stress, muscle stimulation and reperfusion following ischemia (55,182,231,247). Chronic hypoxia, from a deficiency of cellular oxygen delivery, can cause tissue damage in a number of conditions, and reoxygenation following hypoxia is known to result in ROS formation. However, whether ROS are generated during hypoxia, prior to reoxygenation, is controversial. Many people believe that in low oxygen environments, it is difficult to form superoxide (O₂•⁻), the precursor of most forms of ROS. Furthermore, conditions of high oxygen exposure promote the formation of O₂•⁻ as well as its derivatives such as hydroxyl radical and peroxynitrite (225). ROS generation is more likely to occur during reoxygenation since during hypoxia there is a significant accumulation of reducing compounds in mitochondria such as NADH in the
cytosol. The sudden exposure to oxygen promotes the reduction of O$_2$ to O$_2$$^-$. However, Vanden Hoek et al. (231) and Damerau et al. (55) have observed increased ROS production during the hypoxia, prior to reoxygenation, in cardiac myocytes, consistent with the earlier observations by Park et al., using electron spin resonance (169). This is a good example of the paradox of free radical biology, i.e. both low and high oxygen can cause ROS formation (49). Although hypoxia-induced ROS formation has been described during conditions of ischemia or “stimulated ischemia” in cardiac muscle or in isolated cardiac myocytes, it has never been documented in skeletal muscle. Therefore, we tested the hypothesis that ROS are produced in skeletal muscle during hypoxia using fluorescent probes for hydrogen peroxide (H$_2$O$_2$) and O$_2$$^-$ in isolated diaphragm. Our results demonstrate that significant intracellular H$_2$O$_2$ is produced in response to acute hypoxia. However, the results also demonstrate the possible difficulties, pitfalls and artifacts that are unique to the detection of ROS in conditions of hypoxia.

5.3 Methods

Male Sprague-Dawley rats (350–450g) and Swiss-Webster mice (25–35 g) were housed and treated according to approved protocols of The Ohio State University Institutional Laboratory Animal Care and Use Committee. Rats were anesthetized with sodium pentobarbital (~40 mg/kg) or ketamine (~76mg/kg)/xylazine (~15mg/kg), tracheotomized and ventilated. Each diaphragm was removed and dissected into several muscle strips. Each strip was attached to a clear plastic frame using superglue. Prior to experiments, the strips were kept in Ringer’s solution (in meq/L: 21 NaHCO$_3$, 1.0 MgCl$_2$, 1.2 Na$_2$HPO$_4$, 115
0.9 Na$_2$SO$_4$, 2.0 CaCl$_2$, 5.9 KCl, 121 NaCl, 2.07 g/L glucose and 10 µM d-tubocurarine) at room temperature, bubbled with 95% O$_2$ and 5% CO$_2$.

For H$_2$O$_2$ detection, control tissue strips were loaded with 50 µM dihydrofluorescein diacetate (Hfluor-DA, sensitive to H$_2$O$_2$) at room temperature for 60 min, and then were washed with fresh buffer for 10 min (248). Ebselen, a glutathione peroxidase mimic used as a scavenger of H$_2$O$_2$ (206), was applied to the treated tissues at a 40 µM level in the loading solution and throughout the rest of the experiment. For O$_2^{•−}$ detection, hydroethidium (HE) was used as a fluorescent probe (247). The major loading procedures for HE and antioxidant Tiron application (247) followed similar conditions described in chapter 2 of this dissertation.

Fluorophore-loaded tissues were transferred to a superfusion chamber illustrated in Fig. 5.1. In this chamber, > 1 mm of space under the tissue allowed for continuous superfusion of both sides of the tissue. A fiberoptic tissue fluorometer (Radnoti Inc., CA) was used to measure epifluorescence at specific excitation and emission wavelengths. As shown in Fig. 5.2, the excitation beam from a 150 watt xenon lamp was focused and passed through a filter wheel containing four band pass filters. A second filter wheel with four emission filters was used to isolate emission light at a specific range of wavelengths. This light was then focused on the photomultiplier tube (PMT, Model HC 120-05MOD-6308, Hamamatsu PMT Assembly, Japan). Thus, the tissue fluorometer could sequentially measure four channels of fluorescence, i.e., four emission signals from four different excitation signals, as shown in Fig. 5.3. In the time frame of this
experiment, the four channels of fluorescence were recorded simultaneously. For these experiments, the filters selected were in four different ranges: NADH range: Ex 330 ± 40 nm, Em 470 ± 5 nm; Fluorescein (Fluor) range: Ex 490 ± 10 nm, Em 535 ± 17.5 nm; Ethidium Wide (ET W) range: Ex 455 ± 35 nm, Em 630 ± 25 nm; Ethidium Narrow (ET N) range: Ex 470 ± 5 nm, Em 610 ± 5 nm. These four different Ex/Em ranges provided a relatively complete observation window for tissue autofluorescence and the fluorescence of specific ROS probes for \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \). In order to minimize the light interference, the tissue chamber was housed in a solid metal black box. The PMT transduces the emitted light into a greatly amplified voltage, and this electronic signal is sent to a computer by an A/D converter. The fluorescent measurements from each filter set were signal-averaged, recorded and graphed using a program developed by our laboratory in a modified programming language (Workbench PC, Strawberry Tree, Inc., CA). Sampling of the light was limited to a 7 s window every 30 s, and within this time window, the tissue was exposed to a given wavelength of light for about 8.5 ms every 40 ms. The amount of light reaching the tissue was therefore reduced as much as possible to avoid photobleaching and photooxidation of the fluorophore.

Prior to hypoxia, tissues were perfused with Ringer’s solution bubbled with 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \) for 10-15 min or until the fluorescent emission was stabilized. Then hypoxia was induced by superfusing the tissues with buffer equilibrated with 74-95% \( \text{N}_2 \) and 5% \( \text{CO}_2 \) (i.e. 0-21% \( \text{O}_2 \)) for 10-15 min. After hypoxia, tissues were reoxygenated with 95% \( \text{O}_2 \). The switching between oxygenated and deoxygenated buffers was done with a remote electronic switch and a solenoid within the black box. Temperature was
measured and maintained at either room temperature or 37°C using a self-controlled stage heater (Warner Instrument Co., CT) and two heating pads installed in the black box. For calibration purpose, the autofluorescence of each tissue strip was also measured in hypoxia/reoxygenation. The level of O₂ during hypoxia was varied by using superfusate bubbled with 0-30% O₂ and an oxygen mixing system (Reming Bioinstruments Co., NY). Room temperature was preferred in most measurements because at this temperature the leakage of the dye was greatly reduced compared to 37°C. Usually, the rat diaphragm was preferred for these experiments, since the size of the rat tissue strips can usually fit the chamber better and the more strips can be made from each rat diaphragm than mouse diaphragm. However, in some experiments, the mouse diaphragm was used in order to verify the results from rat or test for other particular reasons.

5.4 Results

5.4.1 Tissue autofluorescence in hypoxia

Typical autofluorescence with exposure to varying degrees of hypoxia at 37°C is shown in Fig. 5.4. As expected, fluorescence in the range of NADH (blue) proportionally increased in hypoxia, and in the range of FAD (green, red, brown in channel 2,3,4) proportionally decreased with hypoxia. For clarity, fluorescence intensity is normalized to the NADH initial fluorescence. The 0% O₂-hypoxia exposure provided the largest increase in the NADH signal, while interestingly, 30% O₂ still caused measurable changes in NADH. (Note: in our measurements, the FAD autofluorescence signal may include some contribution from FMN autofluorescence.)
In some experiments, the PO$_2$ in the tissue chamber was measured using a miniature O$_2$ electrode (World Precision Instruments, Inc., FL). The PO$_2$ in the chamber ranged from 2-6% O$_2$, depending on the location of the electrode. This level of O$_2$ probably arose from diffusion of room air at the chamber surface. Because of uncertainties in its measurement, we did not attempt to accurately estimate PO$_2$ in the chamber. In this study, additional development of equipment and technique will be necessary to pinpoint the exact relationship between PO$_2$ and ROS formation. Nevertheless, there was qualitatively little difference in signals between exposures to a wide range of different levels of hypoxia. The grouped data were obtained in the condition of 0% O$_2$ and room temperature (N=6). Each curve is normalized to its average initial fluorescence intensity, respectively, as shown in Fig 5.5.

### 5.4.2 Fluorescence from Hfluor-loaded tissues during hypoxia

The oxidation of Hfluor to fluorescein (Fluor), thought to be largely due to H$_2$O$_2$ formation (248), was evaluated by measuring Fluor fluorescence. These experiments were run at room temperature in this study in order to preserve retention of the dye. However, pilot experiments were also performed at 37°C, which showed similar responses. Typical response patterns show a transient increase in fluorescence in the Fluor channel, followed by rapid and apparent washout during hypoxia as observed at the second channel in both panels of Fig. 5.6. Note, this is opposite to the expected autofluorescence in the same channel (Fig. 5.4 and 5.5).
5.4.3 Inhibitory effects of ebselen on fluorescein spectra in hypoxia

Ebselen is a glutathione (GSH) peroxidase mimic, and works with GSH to scavenge H₂O₂ (132, 206). As shown in Fig. 5.7 (raw data) and 5.8 (grouped data), at the 40 μM level, ebselen completely inhibited the transient rise in fluorescence seen in the Fluor channel during hypoxia. After a 30 min washout of ebselen, the increase in fluorescence with hypoxia returned (data from the same tissue later, Fig. 5.7). Grouped data of comparison between treatment and control are shown in Fig. 5.8. Ebselen scavenging caused a significant reduction of Fluor emission compared to control (N=5, * P < 0.05, Fig. 5.8.), and resembled the expected decrease in fluorescence from the superimposed autofluorescence at these wavelength from the conversion of FAD to FADH during hypoxia (Fig. 5.5).

Two other sets of control experiments were performed in order to better understand the unusual response of the fluorophore to changes in oxygen. The rapid drop in Fluor fluorescence that occurs during the phrase of reoxygenation was particularly puzzling (Fig. 5.6 and 5.8.), requiring future investigation. First, we hypothesized that the changes in fluorescence with hypoxia were a false signal and represented the known influence of O₂ as a fluorescence quencher. However, experiments performed using the fluorometer and a specific cuvette system demonstrated an insignificant O₂ quenching effect. Interestingly, but not relevant to our results, CO₂ was shown to quite significantly quench the Fluor fluorescence (data not shown). A second hypothesis was tested that there is some cellular event that causes the loss of the signal during reoxygenation, such as
intracellular redistribution of the fluorophore. To test this, 5 μM fluorescein-diacetate (Fluor-DA, the oxidized form of Hfluor) was loaded into the muscle and the tissue was exposed to hypoxia, a very similar fall in fluorescence was seen during the hypoxia phase, which resembled the fall seen in the unoxidized probe (data not shown).

5.4.4 Measurements of $O_2^{•−}$ formation with HE

A large number of experiments were performed using the $O_2^{•−}$ fluorescent probe, HE, discussed in the previous section in heat stress (chapter 2). In this experiment, ethidium (the oxidized product of HE and ROS) fluorescence rose sharply in response to acute hypoxia, as shown in the fourth channel of left panel in Fig. 5.9. Like the previous results for Fluor, the ethidium signal rapidly declined during the reoxygenation phrase. The signal was not blocked by the $O_2^{•−}$ scavenger, Tiron (data not shown). Even more interestingly, when the oxidized form of HE probe (ethidium) was loaded into the muscle prior to hypoxia exposure, a very similar response was observed (right panel of Fig. 5.9) compared to HE-loaded tissues. Our inability to completely block the changes in fluorescence with antioxidants and obvious artifacts in the signal led us to abandon HE probe as an adequate measure of ROS in hypoxia.

5.5 Discussion

Hypoxia is particularly common in exercising skeletal muscle due to a large amount of oxygen demand (186), and thus the intracellular oxidant level may be influenced by hypoxia-induced reductive stress shown as the accumulation of reductants such as NADH in the mitochondrial matrix (55,231). In our laboratory, a previous study has shown that
antioxidants have protective roles on the function of skeletal muscle during hypoxia (150). However, the direct evidence of ROS formation in skeletal muscle is not available. The present studies have provided the clear evidence of intracellular ROS production in skeletal muscle, and determined the stimulatory effect of acute hypoxia on intracellular ROS formation, particularly H₂O₂ generation, detected by a H₂O₂-sensitive fluorescent probe. The antioxidant enzyme mimic ebselen, completely abolished the Fluor signal, confirming that the signal arose from the intracellular formation of H₂O₂ in skeletal muscle. The unusual properties of this signal, i.e., the rapid fall during reoxygenation reflects a non-ROS dependent chemical or cellular event that can also be reproduced in experiments with tissues loaded with the oxidized probe. The lack of an antioxidant-blockable signal using the probe HE is interesting and might represent the poor ability of low levels of O₂⁻ to compete with other cellular scavengers such as superoxide dismutase (SOD) and *NO. We hypothesize that the SOD activity in muscles is so strong that it quickly converts O₂⁻ signal to H₂O₂, which can then be picked up by Hfluor.

5.5.1 Effects of hypoxia on tissue autofluorescence
The intracellular levels of NADH and FAD have great impacts on autofluorescence in their respective emission ranges, which extends throughout the visible range. Our results illustrate the critical importance of measuring autofluorescence when using these redox sensitive probes as it can greatly alter the interpretation of the signal. For example, HE has the similar excitation and emission to NADH, and when loading is poor, the emission signal of HE cannot provide reliable information about O₂⁻ formation due to the NADH
interference (247,248). In this study, we varied the O₂ concentration in the perfusate during hypoxia from 30% O₂ to 0% O₂, and the NADH signal decreased relatively proportionally to corresponding increase in % O₂. As shown in Fig. 5.4, the maximum induced NADH signal was observed at 0% O₂, demonstrating that the NADH signal was a reliable indicator of the level of tissue hypoxia. FAD autofluorescence decreased in response to hypoxia, consistent with the fact that hypoxia causes an increase of FADH, which has no fluorescence. These autofluorescence experiments are very necessary before testing tissues loaded with the dye, in these excitation-emission ranges. The results of autofluorescence can be used for calibrating and analyzing the data with fluorophore-loaded tissues, thus avoiding any artifacts (247,248). Similar results can be seen at body temperature (Fig. 5.4) and room temperature (Fig. 5.5).

5.5.2 Detection of hypoxia-induced formation of ROS

During hypoxia, the buildup of reducing equivalents in the mitochondria such as FADH, NADH and reduced CoQ, would tend to interact with any available electron acceptors such as oxygen, resulting in the formation of ROS. Fig. 5.6-5.8 show that a significant level of H₂O₂ was quickly generated in response to hypoxia, and this signal can be blocked by the antioxidant ebselen. This is the first report that hypoxia can induce ROS formation in skeletal muscle, and is consistent with previous studies that showed increased ROS production during the hypoxia prior to reoxygenation in other tissues (55,231).
We also used the $O_2^{•−}$ sensitive probe HE (247). In the presence of $O_2^{•−}$, HE is oxidized to ethidium. We later found that hypoxia caused an increase of ethidium fluorescence. However, this signal cannot be blocked by the antioxidant Tiron (data not shown). With the consideration that ethidium is a cation and may possibly be sensitive to membrane potential, we speculated the possibility that the signal may be an artifact due to hypoxia-induced membrane potential change. Therefore, we used ethidium bromide, the oxidized probe of HE, to load into the tissues, and repeated the whole experiments. The results were quite similar to HE-loaded tissues (Fig. 5.9). We speculate two possibilities: 1) HE may not accurately distinguish $O_2^{•−}$ induced by hypoxia, possibly due to the membrane potential artifact; 2) the level of $O_2^{•−}$ that is produced during hypoxia can be quickly dismutated to $H_2O_2$ by SOD, resulting in a very low level of $O_2^{•−}$ that is undetectable by HE.

Furthermore, Fluor, the oxidized probe of Hfluor, was also tested for artifacts similar to those seen with ethidium. The increase of Fluor induced by hypoxia was also observed. But this could be an artifact because it couldn’t be blocked by the ebselen, the same antioxidant that did block the signal when the unoxidized probe was used. These data suggest that the mechanism for this artifact may be totally different from the former observation with Hfluor, and thus requiring further studies.

5.5.3 Biological effects of ROS induced by hypoxia

Cellular hypoxia plays a critical role in many biological systems. Regarding cell signaling events, hypoxia can activate transcriptional regulators including nuclear factor-
κB, hypoxia-inducible factor-1, activator protein-1, and mitogen-activated protein kinases (MAPK) (131). Particularly, these effects from hypoxia may be mediated by ROS. Kulisz et al. have shown that hypoxia activates p38 MAPK phosphorylation through increasing hypoxia-induced ROS formation by mitochondria (125). Furthermore, mitochondrial ROS formation induced by brief hypoxia can initiate significant preconditioning effects for the protection of cardiomyocytes (230).

Hypoxia can also affect a number of important enzymes such as mitochondrial enzymes as well as antioxidant enzymes (131). The activities of most important mitochondrial enzymes (including citrate synthase, malate dehydrogenase, Complex IV and MnSOD) can all be reduced during hypoxia (131). Since mitochondria are believed to be one of the major ROS generators, any influence on them may possibly modify the intracellular redox status. Hypoxia also decreases the function of other antioxidant enzymes including catalase and glutathione peroxidase, apparently reflecting a possibility that this may greatly affect the intracellular oxidant level (131).

5.5.4 **Sources of hypoxia-induced ROS formation**

Hypoxia appears to be related to the production of ROS either directly or indirectly, and the mitochondrion seems to be a candidate for ROS generation during hypoxia (125,131,230) as mentioned in section 5.4.3. Xanthine oxidase (XO) is another common ROS generator, and could be particularly important in hypoxia/anoxia and ischemia-reperfusion injury. This is because during hypoxia, XO can be phosphorylated by hypoxia-activated p38 MAPK, and the phosphorylation is critical for the full activation of
XO (131). The activities of XO can also be elevated by an excess Ca\(^{2+}\) influx induced by hypoxia (25). However, the exact role of these two major ROS generators in hypoxia-treated skeletal muscle has not been identified. Therefore, future research is necessary to explore their role by applying corresponding inhibitors.

5.5.5 Methodology of epifluorescence with tissue fluorometer

There are a number of ways to measure fluorescence in intact tissues. In chapter 2, we used laser scan confocal microscopy (LSCM) to detect intracellular ROS formation. The most powerful feature of LSCM is its ability to obtain high-resolution images at a cellular level. However, LSCM can only focus on a range of a few cells, and this results in three major disadvantages: 1) even a minor horizontal or vertical movement of the living tissue results in an out-of-focus image; 2) only a few cells can be observed at a time, and if not careful, this may result in biased observations; 3) LSCM uses a very intense laser beam which can greatly photobleach the dyes and photodamage the cells.

Tissue fluorometry turns out to be necessary because it can overcome these limitations associated with LSCM. It has three major advantages: 1) it can be easily used to obtain fluorescent signals from thousands of cells simultaneously because the diameter of its detection platform is hundreds of times larger than LSCM; 2) due to the large scale of observation, most artifacts due to tissue or cell movements can be greatly minimized; 3) the light source has only minimal effects of photobleaching or photodamage. Therefore, the tissue fluorometer can be used as an effective tool, particularly for those who are willing to make a sacrifice regarding details at sub-cellular levels.
A common problem for superfusing living tissues in a chamber is the lack of perfusate underneath the tissues, resulting in a potential for creating a hypoxic environment in the region closest to the objective or detector. We overcome this problem by using a superfusion system underneath the tissue. Another concern of using the tissue fluorometer is related to how deep the excitation light can reach into the inner cell layers or how much the emission signal from intracellular organelles can reach the PMT. Unfortunately, there are no definite answers to these concerns. Some people believe that we should only observe emission signals at these wavelengths in the outer 50 μm of the tissue surface region, based on theoretical but approximate calculations regarding the light scattering. However, our technique has demonstrated that we can observe the best fluorescent signals from light passing through the entire tissue (~ 400 μm thickness), even in the NADH fluorescent range. In these experiments, we changed the system such that excitation light was focused on one surface of the tissue and the emission fiberoptic cable was focused on the opposite surface (i.e. 180°). This suggests that the usual calculations of tissue penetration which are generally used for microscopy do not apply to tissue fluorometry. We believe this is due to the fact that the fiberoptic cables which function as light collections have the capacity to collect photons at a variety of angles, whereas unfocused emission light is usually lost in microscopy. We are currently working on a theoretical understanding of this phenomenon. We should be also aware that the fluorescence at longer wavelengths, such as those signals observed in the FAD channel and ET channel (Fig. 5.3 and 5.4), can penetrate tissues well, while the emission at shorter wavelength, such as NADH channel, has a relatively less ability to penetrate
the tissue. Therefore, the quantification of this wavelength-dependent fluorescence becomes complicated.

5.5.6 Summary

This chapter has provided a direct evidence of hypoxia-induced ROS generation in skeletal muscle, using tissue fluorometry. The pitfalls associated with this technique have also been discussed in great detail. We conclude that tissue fluorometry is an effective tool to explore the mechanism of ROS generation in hypoxia as well as other oxidative stresses such as heat stress (246). However, those artifacts limit its application, and other techniques such as LSCM and electron spin resonance should also be considered as alternative tools for these studies.
Fig. 5.1. Superfusion chamber for fluorescent studies. Left panel: a view from a vertical angle (i.e. right above the chamber). Right panel: a view from a horizontal angle.
Fig. 5.2. Basic system for tissue fluorometry.
Fig. 5.3. Excitation/emission spectra. Top: Typical hypoxia-sensitive endogenous fluorophores, NADH (blue) and FAD (red). Bottom: Two oxidized fluorescent probes used as indicators of ROS, ethidium (red) and fluorescein (green) used. Approximate excitation and emission bandwidths for the filters used can be seen in the center.
Fig. 5.4. Autofluorescence changes during various exposures to different concentrations of O₂.
Fig. 5.5. Grouped data of tissue autofluorescence responding to hypoxia (N=6). Data were grouped every 5 min and normalized to their initial fluorescence.
Fig. 5.6. Fluorescence in tissues loaded with Hfluor during hypoxia. The tissue was exposed to various concentrations of O₂ in order to explore the relationship between PO₂ in the superfusion chamber and the apparent ROS signal. The tissue used in the right panel is a separate experiment from the tissue used in the left panel and is to illustrate the availability in the shape of the transient rise in fluorescence in the second channel (Fluor channel) during hypoxia.
Fig. 5.7. Inhibitory effect of ebselen on fluorescein spectra in hypoxia. From the second channel, ebselen completely inhibited the hypoxia-induced increase of fluorescence. Note that after 30 min washout of ebselen, hypoxia-induced increase of fluorescence seemed to return in the same channel.
Fig. 5.8. Grouped data of a single series of experiment and comparison between Hfluor-loaded tissues in the presence and absence of ebselen during hypoxia. * P< 0.05 between treat and control (N=5 animal). The ebselen treated tissues behaved in a similar way to normal autofluorescence.
Fig. 5.9. Hypoxia experiments were repeated after loading with HE (left panel) or ethidium (right panel), respectively. Acute hypoxia caused the similar increase of ethidium signals in both panels, suggesting that significant artifacts may be generated when HE is used as a ROS probe in this condition.
CHAPTER 6

DETECTION OF REACTIVE OXYGEN AND NITROGEN SPECIES IN TISSUES USING REDOX-SENSITIVE FLUORESCENT PROBES

6.1 Introduction

Many methods are currently available or are emerging for the detection of reactive oxygen and nitrogen species in tissue. Few methods have the appeal of fluorescent imaging techniques, which have the potential for semi-quantitative detection within individual cells. However, despite their widespread use, there are significant limitations to their application that are very often overlooked and can lead to erroneous conclusions. In this chapter, we have outlined common methods of use for these probes with discussion of their individual strengths and pitfalls.

The most frequently used fluorescence probes for measuring reactive oxygen species (ROS) and reactive nitrogen species (RNS) are summarized in Table 6.1, along with their relative sensitivities to a variety of intracellular redox signals. It is important to realize that none of the available fluorescent probes for ROS are particularly quantitative or specific when used in living cells and tissues. The specificity of the RNS probes may be
somewhat better, as will be discussed later. The loss of quantitation of both RNS and ROS fluorescent probes arises from unpredictable loading, varying intracellular retention, heterogeneity of intracellular localization (130), the varying penetration of excitation and emission light, parallel chemical reactions that may arise from peroxidases or other redox-sensitive intracellular elements (96), varying amounts of photobleaching and photo-oxidation (142), additional ROS production from the oxidized forms of the probes (189) and common nonlinearities of many fluorescent detection systems. Nevertheless, fluorescent detection has proven invaluable in countless experiments for qualitative and rough quantitative estimates of ROS and RNS activity at the cellular level using estimates of “relative changes” in fluorescence. Critical to this approach however, are careful standardizations of experimental conditions between experiments and controls, including handling of the tissue, loading procedures and adjustment of the gain of the fluorescent detection systems to a common standard. Whenever possible, it is also advisable to 1) repeat experiments with multiple probes that have different spectral characteristics and specificities or preferably run parallel experiments with completely different ROS or RNS detection methods; 2) Run positive controls with intracellular ROS producing agents such as menadione (96) or nitrazepam (38); 3) Repeat exact experimental paradigms without the fluorescent probe in question to resolve erroneous data due to tissue autofluorescence, particularly if probes are used with excitation and emission wavelengths in the region of NADH fluorescence (47) and 4) repeat control experiments in the presence of antioxidants, ROS scavengers or nitric oxide synthase inhibitors, where appropriate.
Fluorescence data have most often been expressed as the fractional change or absolute output voltage of the fluorescence signal from the “baseline” fluorescence, measured following loading. Therefore common units are “percent change” and “relative units.” The absolute values of these measures have no particular meaning when compared across experimental models, paradigms or detection devices but when averaged together under identical experimental conditions, they can provide some approximation of grouped responses for comparison. At the present time, none of the probes provides the opportunity for a ratiometric determination such as the methods which exist for pH- or Ca$^{2+}$-sensitive probes. Though far beyond the scope of this review, a variety of techniques have been used to monitor the fluorescence changes with these probes. Some examples include reflectance fluorometry (3,246), epifluorescence fluorescent microscopy (18,154), flow cytometry (140) and extraction of oxidized probes from the tissue followed by fluorometry (3,157), HPLC with fluorescence detection (101), and laser scan confocal microscopy (247).

6.2 General guide to loading probes into cells and tissues

Some important initial points can be applied to all the fluorescent probes discussed in this review. First, the final intracellular dye concentrations after loading are generally much greater than the dye concentrations used in the extracellular environment during loading. This occurs due to intracellular compartmentalization or to conversion of the loading form of the probe to a new species, resulting in a sustained concentration gradient across the cell membrane. The clearest example of this applies to the acetoxymethyl ester (AM) or diacetate (DA) forms of certain dyes, which are used to improve penetration of
the cell membrane. Once inside the cell, these attached groups are cut off by intracellular esterases resulting in entrapment and accumulation of the dye inside the cell. The use of a minimal loading dose, resulting in a relatively strong fluorescent signal is recommended. If too much fluorescent dye is loaded into the tissue, the probes can work as either antioxidants or pro-oxidants, disturbing the intracellular environment. The leakage of dyes may also be increased during the experiments due to the overloading. Therefore, careful adjustment of loading concentrations in specific experimental conditions is imperative and must be determined for each model and each new probe.

6.3 Use of hydroethidine for ROS detection

6.3.1 The reaction of hydroethidine with ROS

\[
\text{Hydroethidine} + 2\text{O}_2^- + 3\text{H}^+ \rightarrow \text{Ethidium} + 2\text{H}_2\text{O}_2
\]

6.3.2 Description and general sensitivity to ROS

Hydroethidine™ (HE), or dihydroethidium, is an uncharged hydrophobic molecule,
readily taken up by living cells or tissues (Molecular Probes, Inc. Eugene, OR; Polysciences, Inc., Warrington, PA). It is very sensitive to superoxide (O$_2^-$) (3,13,18), and can be oxidized to ethidium (ET) as shown in Equation I. It is very insensitive to H$_2$O$_2$ (3,13,18). In the process of oxidation, it forms one or more free radical intermediates (not shown) that may be important for some secondary reactions (13). Although some in vitro experiments have demonstrated that it can also be oxidized by *OH to ET (3,231), others have not been able to show this (18). Regardless, this is probably not quantifiable, in vivo, because of low reaction probability and the fact *OH simultaneously reduces the fluorescence of ET when intercalated with DNA (231). Some studies have reported that the probe is insensitive to peroxynitrite (ONOO$^-$) or HOCl (18). However, others have found that HE is easily oxidized by ONOO$^-$, using the ONOO$^-$ generator, SIN-1 (3). One research group has suggested SIN-1 may oxidize HE by separate formation of O$_2^*$ (18). Unlike the fluorescein and rhodamine-based probes, discussed below, HE oxidation is not sensitive to and does not require peroxidases such as horseradish peroxidase (HRP) (40), but it has a small sensitivity to xanthine oxidase (18). ET apparently does not appreciably auto-oxidize, thus avoiding additional production of O$_2^*$ (13), although this may require further evaluation in the presence of cellular reducing reactions (189). As shown in Equation I, the oxidized product is positively charged, contributing to its tendency to accumulate in the nucleus and intercalate with the negatively charged DNA phosphate backbone as well as in other negatively charged compartments such as the mitochondrial matrix and inner cell membrane.
6.3.3 Procedure for loading HE

With a poor solubility in water, HE stock must be made in organic solvent such as dimethyl sulfoxide (DMSO) or N,N-dimethylacetamide (DMAM). Based on our experience, DMAM is preferred for this probe as the stock stays in aqueous phase at -10 to -20 °C, whereas HE-DMSO stock is frozen at 3-4°C. In general, it is advisable to avoid repeated freeze-thaw cycles with these probes. There are other considerations when using DMSO as a solvent. It is a well-known •OH scavenger that could possibly attenuate HE oxidation. However, for this reason it might also aid in separating HE oxidation via O$_2^{•−}$ vs. oxidation from •OH.

The exact concentrations and incubation times for staining in whole tissue preparations should be optimized for each experimental model. However, one recommended procedure (Polysciences, Inc., Warrington, PA), appears to work well for a large range of experimental conditions from cell preparations (30) to whole tissues (247).

6.3.3.1 Stock and loading solutions

7 mg HE is dissolved in 1 ml DMAM and sealed in –10 to -20°C. Fresh loading solution should be made each day. We normally use 20 µl HE stock/10 ml buffer (44 µM HE solution) as regular loading condition. Buffer can be physiologic salt solution for tissues or PBS containing Ca$^{2+}$ for cell preparations (30). Successful loading has been reported for cell preparations as low as 2 µM HE (140). The loading solution should be reasonably clear to light pink, prior to loading. As the stock or loading solution ages,
auto-oxidation of the HE to ET is apparent by the change to a darker pink and ultimately to a red color. This increases the background fluorescence and can thus decrease the sensitivity of HE. In whole tissue experiments loading concentrations of up to 88 μM HE may be necessary for adequate signal intensities.

6.3.3.2 Loading procedure

Successful loading of the HE dye does not appear to be extremely temperature-dependent. However, at times it is convenient for preservation and oxygenation of thick tissues to load in buffer, on ice. Cells or well-perfused preparations can load sufficiently within 15-30 min at 37°C (3,140). Our laboratory loads isolated diaphragm preparations for periods of 1 h, in the dark, in physiologic saline, on ice (247). This is followed by 10-15 min of rinsing in oxygenated buffer. With these loading conditions there remains a variable level of baseline ET fluorescence, prior to initiation of the experiment, presumably due to auto-oxidation HE, or oxidation caused by basal ROS production (247).

6.3.3.3 Precautions

Although HE is relatively nontoxic to cells during the course of most experiments, it is very cytotoxic after extended periods and easily absorbed by the skin and other tissues, combining with DNA as ET. Positively charged ET is also absorbed, and is considered toxic. Care should be taken in handling and disposing of all loading solutions and tissues to completely avoid skin exposure, with appropriate disposal of solutions. Additional
care should be taken with loading solutions that are being bubbled with O$_2$ as these can produce aerosols in the immediate environment.

### 6.3.4 Detection

Theoretically, HE/ET can be used as a dual fluorescent probe, i.e., the loss of HE can be monitored as a blue signal as it is dehydrogenated to ET, a red emission (3). Although in some isolated cell preparations intracellular HE is relatively stable for hours (192), in other tissues such as isolated skeletal muscle at 37°C (247), significant leakage begins to occur after about 15 min. The HE excitation occurs at 360-380 nm and emission is at 430-460 nm, the latter depending on the specific excitation wavelength used and the cell or tissue microenvironment. Furthermore, HE shares similar excitation and emission ranges as NADH (74), a major source of autofluorescence. The short excitation (high energy) wavelengths can also promote photo-oxidation of the probe. These factors limit, somewhat, the usefulness of HE detection as an indicator of ROS formation, but do provide a useful method for monitoring HE loading. In contrast, ET is easily trapped by negatively charged organelles or it can be dissolved well in aqueous cytosol, reducing its membrane penetration. Once formed, it is extremely stable in the cell. Importantly, tissue autofluorescence is weak at ET excitation and emission ranges, light penetration is better because of the longer wavelengths at lower energy, and there is good separation between excitation and emission wavelengths, allowing for a variety of filter options. Therefore, the measurement of relative increases in ET fluorescence has been preferred in most studies to decreases in HE or changes in the ET/HE ratio as an indicator of ROS (3,18,31,157,247).
The excitation wavelengths for ET are actually very broad (3), usually in the 460-490 nm range, but can even be stimulated with an argon/krypton laser line of 568 nm (247). It is worth mentioning that in practice, the excitation and emission wavelengths of most probes are not fixed and depend on numerous variables including the buffer solutions, pH, the thickness of the tissue or even the characteristics of the excitation light and the fluorometer. For example, in reflectance tissue fluorometry, using isolated diaphragm, we have found that both HE emission and ET emission shift to ~ 10-30 nm longer wavelengths than are measured in vitro in solution. This “shift” strategy can sometimes be used to advantage, avoiding the background noise that arises from the Raman scatter of H₂O molecules. Therefore, when possible, pre-testing of the optimum excitation/emission spectra for the best signal is highly recommended for each new experimental preparation. The long wavelength emission spectra for ET and the low autofluorescence in this range allow for the use of long pass filters (LP 590).

Although HE/ET is one of the more specific and quantitative of the fluorescent probes for ROS, it still shares some of the limitations of other more commonly used probes. For example, HE can be oxidized by ferricytochrome c but this results in a new product, distinct from ET (13). The fluorescence properties of this product are not well described. HE also catalyzes the dismutation of O₂⁻⁻, thus diminishing the ET signal in the presence of high concentrations of O₂⁺⁻ (13). This may not be relevant under most intracellular conditions, because HE does not compete well with superoxide dismutase (SOD) or nitric oxide *NO (40).
The accumulation in negatively charged compartments and the binding to DNA/RNA has some additional effects on the ET signal. For example, with DNA binding, there is a 20-fold increase in quantum efficiency of the ET fluorescence signal, measured in vitro (130). However, it is possible that this may be countered somewhat, in vivo, by the influence of the histones, preventing ET from fully accessing the DNA and thus possibly influencing the emission signal (unpublished). The net effect of these influences is difficult to quantify but is certain to result in a very nonlinear output signal. The tradeoff, however, is the stability of the accumulating signal in the cell, which can even be measured in preserved tissue (3,18). Another complicating feature is the possibility that ET accumulation in mitochondria can act as an indicator for the mitochondrial membrane potential, i.e., ET fluorescence is influenced by the depolarization of the membrane (31). For this reason the lowest concentration of HE should be used which still provides an adequate image or signal.

6.4 Use of fluorescein- and rhodamine- based ROS probes

6.4.1 General description and sensitivity to ROS
Fluoresceins and rhodamines, which share a similar structure, are highly fluorescent compounds, but when reduced to their dihydroxyl forms (Fig. 6.1), they exhibit little or no fluorescence. The reduced derivatives of fluorescein are normally attached with diacetate (DA) for better penetration into cells or tissues (Fig. 6.2), but this is not necessary for dihydrorhodamine 123. Once the diacetate molecules get into the cytosol,
intracellular esterases cut off the acetate groups, resulting in better retention within the cytosol. Oxidation in the cell results in conversion to fluorescent species. Four commonly used probes are illustrated in Fig. 6.1.

In general, these probes have been considered indicators of the presence of H$_2$O$_2$ (2,9,34,106,182,191,223). However, in recent years their potential reaction with a number of other oxidative enzymes and intermediates and their ability to self-propagate free radical reactions have resulted in some skepticism regarding their specificity for endogenous H$_2$O$_2$ (142,189,190). This is discussed in more detail, below. Our laboratory shares the view of Hempel et al. (96) that these probes “act as detectors of a broad range of intracellular oxidizing reactions.” The probe, 2’’,7’’ - dichlorodihydrofluorescein (DCFH), is by far the most utilized in biological systems and the most studied with regard to its oxidation-reduction pathways. It will be discussed later as a general model for fluorescein-like probes and a standard for comparison of the behavior of the other probes illustrated in Fig. 6.1.

6.4.2 2’’,7’’ - Dichlorodihydrofluorescein (DCFH)

The sensitivity of DCFH to oxidation by H$_2$O$_2$ was demonstrated in 1965 by Keston et al. (106). The presence of some form of a peroxidase enzyme is required in the H$_2$O$_2$ reaction for all DCFH-like probes as illustrated in Fig. 6.2. None are appreciably responsive to H$_2$O$_2$ alone (96). Zhu et al. showed that iron/ H$_2$O$_2$-induced oxidation could be prevented by the H$_2$O$_2$ scavenger, catalase, or the *OH scavenger, DMSO, suggesting that DCFH is oxidized by both species (245). The probe is clearly not
sensitive to $\text{O}_2^{\cdot\cdot}$ (245). Importantly, it is capable of spontaneous oxidation with peroxidase enzymes, SOD, catalase and lipoxygenase (96) and can serve as a substrate for xanthine oxidase (XO), competing with hypoxanthine (96, 245). These findings regarding DCFH may present problems with interpretation, for example, in conditions of ischemia where XO may be activated.

An important but generally unappreciated observation is that DCFH is sensitive to nitric oxide ($\cdot\text{NO}$) in the presence of $\text{O}_2$ (54, 96, 154), a reaction that may be unique compared to other fluorescein-based ROS dyes (96), Table 6.1. The exact chemical reaction is not entirely clear, but it likely involves other redox forms of $\cdot\text{NO}$ such as nitrogen dioxide ($\cdot\text{NO}_2$) (54). Of note, not all investigations have come to this conclusion (118). In cultured neuronal cells it has been estimated that ~ 50% of the DCFH fluorescence could be attributed to $\cdot\text{NO}$ (88). Peroxynitrite strongly oxidizes DCFH and requires no other cofactors (54, 96). Iron ($\text{Fe}^{2+}$), in the presence of $\text{O}_2$, has a relatively slow oxidizing effect on DCFH, possibly because of its capacity to independently generate ROS species (177). This may be true, particularly in the presence of ascorbate, thus keeping $\text{Fe}^{2+}$ in the reduced form (96).

6.4.3 Dihydrofluorescein (HFLUOR)

Dihydrofluorescein (HFLUOR) is one of the oldest fluorescent probes and was recently re-evaluated by Hempel et. al (96), who found it to have some superior characteristics to DCFH, DHR123 and 5&6CDCFH (Fig. 6.1). Its oxidized product, fluorescein, has a considerably higher molar fluorescence (i.e. highest molar extinction coefficient at 490
nm) and probably more importantly, much better intracellular loading compared to any of the other probes. Our own experience in isolated skeletal muscle has confirmed that it results in very high fluorescence intensity in isolated diaphragm muscle (unpublished). By comparison with DCFH, HFLUOR has a similar sensitivity to HRP, a reduced sensitivity to Fe$$^{2+}$$, even in the presence of ascorbate, a reduced sensitivity to SOD, catalase, lipoxygenase and XO, and is essentially unresponsive to cytochrome c and •NO (96). It is oxidized by peroxynitrite, but at a lower rate compared to DCFH or DHR123 (96).

6.4.4 Dihydrorhodamine 123 (DHR123)

DHR123 also has similar reaction characteristics as DCFH. The diacetate form is not used because DHR123 already has good cell penetration. Like DCFH, it is thought of as a H$_2$O$_2$ probe (43,80,191). However, it is sensitive to ONOO$^-$ (54,96), lipoxygenase activity, xanthine oxidase activity but not to •NO (96,119). DHR 123 can also be used for staining mitochondria (80,211) because the oxidation product, rhodamine 123 (Rh123), is positively charged, making it preferably bind the negatively charged mitochondrial matrix. This has certain advantages in some experiments where there is a need for improved cellular retention of the oxidized product (191). However, it can also cause problems with interpretation, as discussed below.

6.4.5 5-(and6)-carboxy-2',7'-dichlorodihydrofluorescein (5&6CDCFH)

5&6CDCFH has been less frequently used in studies of oxidative stress compared to DCFH or DHR123. It is part of a series of DCFH derivatives designed to improve
cellular retention. This group also includes a diacetoxyethyl ester version and a chloromethyl DCFH-DA version (Molecular Probes, Inc., Eugene OR). Unfortunately, little independent information is available regarding the reaction characteristics of these probes. However, Hempel et al. (96) compared 5&6 CDCFH to the other probes listed in Fig 6.1 and found that it had a low fluorescence intensity in cell culture, but also a lower spontaneous oxidation in the presence of HRP, XO and other enzymes and the lowest sensitivity to iron or cytochrome c, but comparable responses to H₂O₂ in the presence of peroxidase. It was not sensitive to *NO and similarly responsive to ONOO⁻ compared to HFLUOR. It is possible that this probe may have some advantages by providing a low background signal while maintaining good cellular retention and good sensitivity to H₂O₂ (96). However, to our knowledge, this has not been thoroughly tested in variety of tissues or cell cultures.

6.4.6 Procedure for loading

6.4.6.1 Stock solutions

DHR 123 and the diacetate forms of the fluorescein-based probes must be prepared in organic solvents prior to dissolving in buffers (Molecular Probes, Inc., Eugene OR). For fluorescein-based probes, many investigators have preferred using stock solutions of 10 mM-33 mM in ethanol (182,191,218). These are stored in the dark at –20° C and often purged with N₂ to prevent auto-oxidation (191). Of note, diluting 10 mM stock in pure ethanol into a buffer will result in an extracellular environment of 0.1% ethanol if the tissue is incubated at 10 μM and 0.4% at 40 μM. As a comparison, in many states within
the U.S., the legal alcohol limit for driving is 0.08%. Therefore, it is important to realize that this level of alcohol could have a significant influence on the biology of the cells in question. This may be particularly relevant since alcohol can promote oxidant formation in some tissues (141). Therefore, care should be taken to treat control tissues with the same vehicle concentration. Our laboratory prefers using DMSO to dissolve HFLUOR and related probes. For example, for HFLUOR we use 10 mg/100 μL DMSO stock. We then load the tissue with a 20μM solution. This maintains the total DMSO in the bathing solution far below the concentration that has any measurable effects on skeletal muscle function (184). Although the use of DMSO could underestimate ROS formation, it is unlikely to give false positive results. If necessary, for some probes it is possible to use a low-toxicity dispersing agents to facilitate loading and possibly reduce the concentration of the DMSO or ethanol (Pluronic® F-127, Molecular Probes, Inc., Eugene OR). For DHR 123, most recent studies have used the methods of Royall and Ischiropoulos (191). A stock solution of 28.9 mM DHR 123 is made in dimethylformamide and stored at −20°C in the dark. Storage vessels are generally purged with He or N₂.

6.4.6.2 Loading solutions and procedure

As with HE/ET, for each experimental preparation, it is best to experiment with different loading concentrations and times with the object to keep the loading concentration as low as practical. Most cell culture studies have loaded these probes at concentrations 5-20 μM for 20-30 min in room temperature or at 37°C in the dark (4,40,96,191,218,231). However, whole perfused or unperfused tissues require higher concentrations for diffusion and as much as 50 μM for periods of one hour at room temperature have been
used successfully (154). These procedures are generally followed by a 15-30 min of rinsing in fresh, oxygenated buffers.

Temperature is a critical variable for all diacetate-linked probes, as the activity of the esterases necessary for cleavage are highly temperature dependent. However, one recommended procedure for all diacetate-loaded fluorophores is to load first at low temperature (on ice) allowing accumulation of the probe into the cytosol. This is followed by incubation at room or body temperature to activate the esterases. This procedure has been effective in poorly vascularized tissues or organs, as reviewed in ref. (23).

6.4.6.3  Precautions

In organic solvents, all of these probes can be absorbed by the skin, so precautions should be followed when handling the loading solutions. However, any dangers of exposure to these compounds, if they exist, have not been well described.

6.4.7  Detection

Unlike the HE/ET probe, the reduced forms of the fluorescein-like probes do not fluoresce. Therefore, it is not possible to fully estimate the degree of loading of the reduced probe, except by extraction after the experiment (4,191). For testing and calibration purposes, it is sometimes desirable to perform chemical hydrolysis to remove the diacetate. This can be done by treatment with 0.01N NaOH at room temperature for
20 min and readjusting the pH back to physiologic values or by exposing the probe to purified esterases (154).

All of the probes listed in Fig. 6.1 have similar excitation waveforms and can be excited between 480-500 nm. Emission waveforms can be detected at 510-560 nm, or at LP 515 (Table 6.1). Peak emission and excitation wavelengths for HFLUOR, in vitro, are shifted approximately 10 nm lower than the other probes (96), but this may not greatly affect the use of the probe with more common filter sets. Since the excitation and emission wavelengths appreciably overlap for these probes, it is important to ensure that emission filter systems completely block out the excitation light to minimize background noise. One effective combination, used for confocal measurements, is excitation with the 488 nm line of an argon laser. Emitted light can then be collected with a > 515 nm long pass filter (218).

These probes have a very strong tendency to photo-oxidize and photobleach, particularly when excited with laser light and this can, by itself, produce ROS (142). Therefore, every attempt should be made to limit the time of light exposure and data collection. Procedures can be used to estimate the extent of photobleaching and photo-oxidation as described by Murrant et al. (154).

The oxidized DHR 123 (Rh 123), having a positive charge, tends to have good cellular retention, possibly because of accumulation in the mitochondria (191). Most investigators have utilized DCFH at or below room temperature and have demonstrated
good retention of the reduced probe for periods of an hour or more (154,218). At higher
temperatures, 37°C or above, our experience has been that 5&6CDCF is poorly retained
in diaphragm cells but FLUOR is retained relatively well at these temperatures
_(unpublished)_). There appear to be large differences in the leakage between various cell
types as endothelial cells leak DCF rapidly but cardiac myocytes do not (218). In
myocytes it appears that DCF may also concentrate in mitochondria, thus contributing to
retention (218).

One of the major difficulties in interpreting the results from experiments using any of the
fluorescein- or rhodamine-based redox probes is the apparent lack of specificity of the
reactions. Much of this has been described in recent years by Mason and colleagues
using electron spin resonance techniques to identify the intermediate reactions
(142,189,190). The pathway for the reaction of dihydrofluorescein (HFLUOR) with
H₂O₂ and peroxidase is illustrated in Fig. 6.2. This is based on the work of Rota et al.
(189), in studies on DCFH, assuming that the reaction is essentially the same for
HFLUOR, since the reaction characteristics with HRP are similar. H₂O₂ reacts with
peroxidase enzymes to form an enzyme intermediate and in the process oxidizes the
DCFH to the semiquinone free radical, DCF⁺⁻. This is further oxidized by O₂, forming
new O₂⁺⁻ and DCF. Thus, DCFH oxidation is inherently autocatalytic (189). However,
there are other sources of ROS in these reactions: 1) The oxidized form, DCF, can
undergo new reactions with a peroxidase and H₂O₂ to form a new phenoxyl radical,
which has the same valence but comprises a different molecular species from the
semiquinone in Fig. 6.2 (190). When formed in the presence of reducing compounds
such as glutathione (GSH) or NADH, O$_2^\cdot$ is again generated (190). 2) Photooxidation of DCF in the presence of NADH also produces additional O$_2^\cdot$ (142). Thus, we have numerous sources of self-propagation of the ROS when using this category of probe. This tendency to self-amplify the reactions could lead to erroneous conclusions in conditions in which cell injury or decompartmentalization of peroxidase enzymes or probes could generate very nonspecific increases in fluorescence.

One important question which remains to be addressed is exactly what peroxidase or oxidase-like enzymes are important in these reactions in the cell and are they ever rate limiting? For example, the fact that xanthine oxidase (XO) can alone oxidize DCFH is troubling because this enzyme is known to be increased in many tissues during conditions of hypoxia, ischemia or ischemia/reperfusion, some of the most studied forms of oxidative stress. Catalase, SOD, lipoygenase all seem to contribute to spontaneous DCFH oxidation. Zhu et al. (245) have suggested peroxisomal-sugar and amino acid oxidases, as well as heme proteins could provide the enzymatic machinery for these oxidations and as cells are stressed, these enzymes could change their activity. Even some antioxidants could affect the oxidation of DCFH to DCF. For example, Trolox, a hydrophilic analog of vitamin E, can oxidize DCFH to DCF through the hydrogen abstraction by the phenoxyl radical of Trolox (105). Therefore, the use of these probes should be interpreted with caution.
6.5 Diaminofluorescein (DAF) probes for measurement of RNS

6.5.1 General description and sensitivity

Recently, Kojima et al. have designed and synthesized a series of novel fluorescence indicators that are derivatives of the diaminofluorescein (DAF), including 4,5-diaminofluorescein (DAF-2) (115) and 3-amino,4-aminomethyl-2',7'-difluorofluorescein (DAF-FM) illustrated in Fig. 6.3 (112,113,156). The DAFs react with *NO, in the presence of O₂, to form triazole derivatives, emitting a green fluorescence with the high sensitivity (5 nM for DAF-2, 3 nM for DAF-FM). This has made it practical in the study of dynamic alterations in *NO of cells or tissues (29,112,135). Both DAF-2 and DAF-FM come in diacetate forms (DAF-DA and DAF-FM DA), which can be employed for better intracellular loading, as discussed earlier. The emission of DAFs was shown to be highly dependent on the intracellular *NO concentration and was not sensitive to O₂⁻, NO₂⁻, NO₃⁻, H₂O₂ or ONOO⁻ (112). DAF-2 has been used in a number of biological studies, providing a fluorescent image with fine resolution (14,72,113,115,135,156). Interestingly, even after aldehyde fixation, DAF-2 loaded cells still leave a record of *NO production before fixation (216). However, one of the problems with earlier DAF analogs, including DAF-2, is that they are pH sensitive resulting in a rapid fall in fluorescence below pH 7.0 (101). DAF-FM shows little sensitivity to pH (116), and can detect *NO, linearly between 3-200 nM (101). It has been suggested that DAF-FM may have some considerable advantages besides its lack of pH sensitivity, including better photostability of the triazole product and greater sensitivity to *NO compared to DAF-2 (101,116).
6.5.2  Procedure for loading

6.5.2.1  Stock solutions

DAF-FM and DAF-FM-DA are supplied in 7 mM and 5 mM solutions of DMSO, respectively, for which it is recommended they be stored at -20°C in the dark (Molecular Probes, Inc. Eugene, OR.). DAF-2 or DAF-2-DA (Sigma, St. Louis MO, or Calbiochem, La Jolla, CA) are supplied as a powder or in a 5 mM solution of DMSO and stored in the dark at -2 to -8°C.

6.5.2.2  Loading procedures

To our knowledge, the DAF probes have not been used extensively in whole tissue preparations. However, the procedures for loading cells using DAF-FM-DA or DAF-2-DA generally employ concentrations of 10 μM in an appropriate buffer (101,135). Cells are incubated for 30 min at 37°C and are then rinsed thoroughly with several changes of buffer for 10 min. Waiting an additional 15-30 min for complete de-esterification of the diacetates has also been recommended (Molecular Probes, Inc., Eugene, OR).

The non-esterified versions of the probes can be used for measuring extracellular release of •NO (101). In this application 1 μM of DAF-FM or DAF-2, in the non-esterified form, is incubated with the cells and aliquots removed and stored on ice for later HPLC measurement.
6.5.2.3  **Precautions**

Handling requires similar precautions to that described previously for fluorescein and rhodamine-based probes.

6.5.3  **Fluorescent detection of *NO**

6.5.3.1  **Detection with DAF compounds**

For intracellular *NO detection using DAF compounds the peak excitation and emission wavelengths are similar to the other fluorescein-based probes discussed earlier, approximately 490 nm excitation, 515 nm emission. Although the Ex/Em wavelengths listed in Table 6.1 are adequate for this probe, band pass filtering of 450-490 nm excitation and 515-560 emission have also proven favorable (135). Laser scan confocal detection can be used as described previously for fluorescein and rhodamine-based probes or following procedures used for DAF-compounds (135). For extracellular detection, reversed-phase HPLC with fluorescence detection has been utilized (101).

These probes are relatively new and have received less independent scrutiny regarding the biochemistry of their reactions than the probes for ROS. The reaction site on the molecule is quite different from that of DCFH or HE as shown in Fig. 6.3, and therefore it is unlikely that the compound forms analogous radical intermediates, though it may form other radical species that may have important, as yet unidentified side reactions. The DAF-probes also appear to be susceptible to reductants like ascorbate, dithiothreitol, 2-mercaptoethanol and glutathione, which can diminish the fluorescence induced by *NO.
Thus, the interpretation of results using DAF compounds may need to take into consideration changes in intracellular reducing conditions (e.g. during ischemia-reperfusion). The use of exogenous reducing agents should be avoided.

### 6.5.3.2 Alternative approaches for •NO detection

DCFH was mentioned previously and has been used in a number of cell types for •NO detection, including neuronal cells (88), rat cardiomyocytes (203), rat skeletal muscle (154) and rat macrophages (100). However, it is very non-specific and really needs to be coupled with applications of •NO scavengers (e.g. reduced hemoglobin) and ROS scavengers (e.g. superoxide dismutase or catalase) (88). Another probe, 4-((3-amino-2-naphthyl)aminomethyl) benzoic acid (DAN-1) was developed by Kojima et al. (114) and has been used for the imaging of •NO in rat aortic smooth muscle cells (114). The probe, 2,3-diaminonaphthalene (DAN) has been used to monitor the sum of nitrite and nitrate, indirect indices of •NO produced, resulting in the formation of highly fluorescent 2,3-naphthotriazole (NAT) (41). More recently, a lifetime-based •NO probe was developed by Barker et al. (8). The cytochrome c', which binds •NO with its heme component is attached with a fluorescent reporter tail whose lifetime immediately changes once the cytochrome c’ “catches” the •NO molecule. They also attached the cytochrome c' to the optical fiber with fluorescent reference microspheres through colloidal gold for the buildup of ratiometric sensors which have linear and fast response with high selectivity to •NO and excellent reversibility (8). Finally, even the fluorescence quenching of pyrene derivatives by •NO has been used to measure the diffusion coefficient of •NO in the
membrane (58). Overall, fluorescent \*NO probes are still in the development stage. DAF-FM appears to be superior among them.

6.6 Summary

The take home message of this review of techniques is that the fluorescent probes for ROS and RNS have great potential in improving our understanding of redox behavior within cells and tissues. However, data obtained from studies using these probes must be expressed in the context of the limitations of the chemistry of the probes in the cellular microenvironment, which may change under different conditions such as cell stress or injury. In most cases, as suggested (96), results should be described in a general context of reflecting an increase in oxidizing reactions within the cell and not as a quantitative measure of the production of a specific oxidant species. It is highly recommended that results be verified, when possible, with alternative fluorescent probes or preferably using alternative methods such as electron spin resonance or other newly emerging technology.
Table 6.1. Commonly used fluorescent probes for detection of reactive oxygen and nitrogen species.

Ex/Em: excitation and emission of the oxidized form of the probes; O, ●, □: respectively, low to high estimates of probe sensitivity to species, based on citation (in brackets).

Probes expressed in their de-acetylated form. Where appropriate, for cell loading the diacetate forms are used (e.g. DCFH-DA).

<table>
<thead>
<tr>
<th>Probe</th>
<th>Ex/Em (nm)</th>
<th>(O_2)^*</th>
<th>(H_2O_2)</th>
<th>*OH</th>
<th>ONOO*</th>
<th>Cyt C</th>
<th>Fe^[1]</th>
<th>*NO</th>
<th>Peroxidases^j</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE/ET^[a]</td>
<td>460-490/ LP 590</td>
<td>● ●● (7,15,9,16)</td>
<td>O (7,15,9,16)</td>
<td>? ●●● (7)</td>
<td>○ (9,16)</td>
<td>○ (7,15)</td>
<td>○ (9)</td>
<td>HRP: ○ (17)</td>
<td>XO: ● (9)</td>
</tr>
<tr>
<td>DCFH/ DCF^[b]</td>
<td>480-500/ 510-560 or LP 515</td>
<td>○ (2,30,29)</td>
<td>● ●● (2)</td>
<td>○ (2)</td>
<td>● (2,30)</td>
<td>○ (2)</td>
<td>○ (2)</td>
<td>○ (2)</td>
<td>HRP: ● Cat: ● SOD: ● GPX: ○ XO: ● LO: ● (2)</td>
</tr>
<tr>
<td>HFLUOR/ FLUOR^[c]</td>
<td>480-500/ 510-560 or LP 515</td>
<td>○ (2)</td>
<td>● ●● (2)</td>
<td>○ (2)</td>
<td>● (2)</td>
<td>○ (2)</td>
<td>○ (2)</td>
<td>○ (2)</td>
<td>HRP: ● Cat: ● SOD: ● GPX: ○ XO: ● LO: ○ (2)</td>
</tr>
<tr>
<td>5&amp;6CDCF/ 5&amp;6CDCF^[d]</td>
<td>480-500/ 510-560 or LP 515</td>
<td>○ (2,30,29)</td>
<td>● ●● (2)</td>
<td>○ (2)</td>
<td>● (2,30)</td>
<td>○ (2)</td>
<td>○ (2)</td>
<td>○ (2)</td>
<td>HRP: ● Cat: ● SOD: ● GPX: ○ XO: ● LO: ○ (2)</td>
</tr>
<tr>
<td>DHR 123/ Rh 123^[e]</td>
<td>480-500/ 510-560 or LP 515</td>
<td>○ (2,30)</td>
<td>● ●● (2)</td>
<td>● (2)</td>
<td>● (2,30)</td>
<td>● (2)</td>
<td>● (2)</td>
<td>● (2)</td>
<td>HRP: ● Cat: ● SOD: ● GPX: ○ XO: ● LO: ○ (2)</td>
</tr>
<tr>
<td>DAF-2/ DAF-2-T^[f]</td>
<td>480-500/ 510-560 or LP 515</td>
<td>○ (44)</td>
<td>○ (44)</td>
<td>?</td>
<td>○ (44)</td>
<td>?</td>
<td>?</td>
<td>○●●</td>
<td></td>
</tr>
</tbody>
</table>

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^a: (Hydroethidine/ Ethidium); ^b: (2', 7'-dichlorodihydrofluorescein/ 2', 7' - dichlorofluorescein); ^c: (Dihydrofluorescein/ fluorescein);
^d: (5-(and 6)-carboxy-2', 7'-dichlorodihydrofluorescein/5-(and 6)-carboxy-2', 7'- dichlorofluorescein); ^e: (Dihydrorhodamine 123/Rhodamine 123); ^f: (4,5- Diaminofluorescein /Triazole form of 4,5- diaminofluorescein;
*(3-Amino-4-(*N*-methylamino)-2',7'-difluorofluorescein/ Triazole form of 3-Amino-4-(*N*-methylamino)-2',7'-difluorofluorescein). Reaction with H$_2$O$_2$ in the absence of peroxidases is essentially nonexistent. Reactivity assumed the same as other DAF-probes.

Peroxidase/oxidase abbreviations: HRP: Horseradish peroxidase; XO: xanthine oxidase; Cat: catalase; SOD: superoxide dismutase; GPX: glutathione peroxidase; LOX: lipoxygenase.

* contradictionary or uncertain findings in literature.

Fig. 6.1. Commonly used fluorescein and rhodamine-based probes for detecting ROS. The acetylated forms, used for loading of the fluorescein probes are not shown. The simple reduced forms are shown in the schematic drawings and subtitles. Beneath each subtitle, in italics, are nomenclatures for the oxidized forms used in the text.
Fig. 6.2. Proposed scheme for the oxidation steps of dihydrofluorescein by H$_2$O$_2$ and peroxidase, based on the studies of C. Rota, C.F. Chignell, and R.P. Mason, *Free Radic.Biol.Med* 27, 873 (1999), using dichlorodihydrofluorescein. Note that superoxide (O$_2^-$) is formed during this reaction.
In this dissertation skeletal muscle has been studied, focusing on cellular responses to stress stimuli that are common to exercising muscle. Of these, heat stress is perhaps the most interesting, because we know so little about it. Yet, it has become increasingly clear that whole body heat stress with or without exercise is an extremely dangerous syndrome, and that heat stroke has become recognized as a major health hazard in high temperature environments (152,209,247). Although this research has been focusing on one tissue, the diaphragm, the work can inspire and perhaps provide insights into the influence of heat stress on other tissues such as cardiac tissue or gut epithelium. Studying the diaphragm has certain advantages that will allow us to make general observations about the cellular responses to heat stress that probably apply to many other tissues. It is of some considerable interest that heat stress may be a “normal” condition for muscle, and this may help us to find some unique adaptive mechanisms of skeletal muscle that don’t exist in other organs. This work on heat stress is fundamental and technically advanced and has inspired new methodologies and understandings of free radical biology as it applies to cellular homeostasis (247). Particularly, this study demonstrated that lipoxygenase (LOX) is responsible for extracellular reactive
oxygen formation. This observation is unique and may lead us to a whole new range of potential preventative strategies for heat stress in susceptible individuals. In addition, it is opening up a new door to our understanding of free radical biology because LOX is not believed to be an important mediator of free radicals or oxidants, though it has been known for decades to have this potential. Secondly, the pathways that provide substrate for LOX, namely phospholipases, are driven by the availability of Ca$^{2+}$, which could be an underlying trigger for reactive oxygen formation in the cells. For example, Ca$^{2+}$ plays a critical role in the conversion of xanthine dehydrogenase to xanthine oxidase, another major ROS generator (25,56).

Considerable interest has been focused on the potential role of ROS in respiratory failure, particularly with respect to its effect on diaphragm, perhaps the most important respiratory skeletal muscle for sustaining life. These conditions are normally related to hypoxia (150,231), sepsis (37), fever (121,172), inflammation (172,239,240) and acidosis (128). Thus, in this dissertation, comprehensive methods were designed to detect ROS from both intra- and extracellular environments. The results showed that heat (42°C) promotes both intra- and extracellular ROS formation. These novel observations were confirmed by abolishing the signals with the intra- and extracellular ROS scavengers. The data also suggest that the well-known upregulation of heat shock proteins may be induced by endogenous ROS formation during heat stress. These techniques are particularly useful for the study of other stresses such as transient and steady-state hypoxia.
The sources of ROS in heat-stressed skeletal muscle were further explored. Current results showed that mitochondria and NADPH oxidases are not major sources of extracellular ROS formation, although they are widely believed to be primary ROS generators in most isolated cell preparations in response to stress. Furthermore, the anion channel, believed to be a major $\text{O}_2^\bullet-$ exit pathway demonstrated from the studies of red blood cells (139) and mitochondria (230), was shown not to contribute to ROS release. LOX, which is upregulated by nitric oxide synthase (NOS)-activated phospholipase A$_2$ (PLA$_2$), appears to be a major extracellular ROS generator. This is extremely important because LOX is closely related to many diseases including inflammation (48,240), atherosclerotic lesions (42), airway disease and autoimmune encephalomyelitis (207). However, its direct role in ROS formation has been largely overlooked. Therefore, LOX inhibition may have a potential for treatment of new varieties of disease, not the least of which is heat shock. Preliminary data suggest that this is not the only source, because intracellular ROS formation is not blocked by LOX inhibition and may represent partitioning of ROS generators between differing organelles in the cell. Therefore, one of my future studies will focus on identifying the intracellular ROS sources. The prime candidates are NADPH oxidases, xanthine oxidases, and NOS. I expect that at least one of the enzymes above will be confirmed to be the primary intracellular source of ROS in heat exposure.

Using a new tissue fluorescence technology, it is possible to identify the kinetics of reactive oxygen formation in the transition from normoxia to hypoxia, the second stress model in this dissertation. The current data strongly show that acute hypoxia caused significant ROS
formation in skeletal muscle. However, these measurements are complicated by dominant autofluorescence, simultaneous leakage and redistribution of the probe during hypoxia, and quenching effect. We speculate that ROS formation during these transient O₂ exposures may have important roles as mediators of O₂ sensation or cell adaptations. Therefore, the future study on hypoxia should be focused on both the source of hypoxia-induced ROS and a more systematic and accurate measurement of ROS with tissue fluorometers, multiphoton confocal microscopy, and other instrumentations.
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