### AN ABSTRACT OF THE DISSERTATION OF

Kristine M. Robinson for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on April 10, 2007.

Title: <u>The Detection of Superoxide and Implications for Amyotrophic Lateral</u> <u>Sclerosis.</u>

Abstract approved:

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Mutations to superoxide dismutase were the first proven cause of Lou Gehrig's disease (amyotrophic lateral sclerosis; ALS) implicating superoxide in the selective death of motor neurons that characterizes ALS. Nitric oxide competes effectively with superoxide dismutase for superoxide to form the powerful oxidant peroxynitrite. Endogenous formation of peroxynitrite can kill motor neurons in vitro and has been implicated in the pathogenesis of ALS in vivo. To further investigate the role of superoxide and peroxynitrite in the pathogenesis of ALS, several new approaches were developed. First, the synthesis of peroxynitrite from nitrite and hydrogen peroxide was simplified to provide a stable source for in vitro experimentation. Second, the products from the peroxynitrite-mediated oxidation of the antioxidant, urate, were determined. Urate is an efficient inhibitor of radicals derived from peroxynitrite without scavenging peroxynitrite directly. Radicals derived from peroxynitrite were found to oxidize urate to the ring-opened product, triuret, which helps explain the previously reported formation of aminocarbonyl radicals from urate. Urate is protective in cell culture models of ALS and in vivo in the treatment of stroke and muscular dystrophy. Unfortunately, oral administration of urate failed to inhibit disease progression in a mouse ALS model.

The third new approach was the development of a method to measure superoxide in vivo, overcoming current limitations of specificity, sensitivity and intracellular access. The assay is based upon the newly discovered reaction of superoxide with the hydroethidine radical to form a transient peroxide that spontaneously decomposes to leave a hydroxyl group. The hydroxyl product can be selectively detected by fluorescence using 396 nm excitation. This novel excitation wavelength is advantageous to the current practice of 500 nm excitation which detects non-specific oxidation. Comparison of fluorescence using both excitations can provide a useful ratio of superoxide-dependent versus non-specific oxidation. Furthermore, covalent modification with the triphenylphosphonium cation targets the dye to mitochondria, providing direct measurement of superoxide in mitochondria. The method we developed revealed that mitochondrially-generated superoxide was increased in astrocytes expressing the ALS-associated mutation, SOD<sup>G93A</sup>. These new tools allow superoxide generation to be measured in ALS models and provide evidence that free radicals killed Lou Gehrig.

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Kristine M. Robinson

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Kristine M. Robinson, Author

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Joseph S. Beckman was involved in the design, analysis, and writing of each manuscript. In Chapter 3, Jeffrey T. Morré assisted with mass spectrometric analysis of oxidation products. In Chapter 4, Dr. Mariana Pehar performed the dissection and culturing of oligodendrocytes and contributed to writing the manuscript. Michael S. Janes performed wide field microscopy experiments. Jeffrey S. Monette isolated rat liver mitochondria and Meredith F. Ross performed triphenylphosphonium electrode experiments. Drs. T.M. Hagen and M.P. Murphy contributed to the design, analysis, and writing of the manuscript. In Chapter 5, Michael S. Janes wrote the wide field microscopy protocol. In Chapter 6, Mark A. Levy assessed SOD activity in astrocytes.

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### LIST OF ABBREVIATIONS

Amyotrophic Lateral Sclerosis (ALS)

Superoxide Dismutase (SOD)

Superoxide (O<sub>2</sub><sup>-</sup>)

Peroxynitrite (ONOO<sup>-</sup>)

Nitric oxide (NO)

Bovine pulmonary aortic endothelial cells (BPACs)

hydroethidine (HE)

ethidium (Et<sup>+</sup>)

2-hydroxy ethidium (HO-Et<sup>+</sup>)

MitoSOX Red<sup>™</sup> mitochondrial superoxide indicator (Mito-HE)

3,8-diamino-5-hexyltriphenylphosphonium-6-phenylphenanthridinium (Mito-

Et<sup>+</sup>)

Hydroxylated Mito-HE (HO-Mito-Et<sup>+</sup>)

Excitation wavelength ( $\lambda_{ex}$ )

Blood-brain barrier (BBB)

2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU)

Chapter 1

Introduction

"Did free radicals kill Lou Gehrig?" Lou Gehrig was known as the Iron Horse of baseball, playing over 2000 consecutive baseball games after joining the New York Yankees in 1925. His batting average was comparable to that of Babe Ruth. In 1938, Gehrig began making simple fumbling errors on the playing field and his batting average fell. Lou Gehrig was diagnosed with amyotrophic lateral sclerosis, a fatal neurodegenerative disease involving the rapid loss of motor neurons which leads to paralysis and death within 2-5 years. Lou Gehrig passed away in 1941, leaving the legacy of a baseball hero and a farewell speech, still considering himself "the luckiest man on the face of the earth." ALS is now more commonly known in the USA as Lou Gehrig's disease. Unfortunately, the cause of ALS is still largely unknown, except that in a small percent of cases it is associated with mutations to the enzyme, superoxide dismutase (SOD; 1). Ninety percent of ALS cases are sporadic, however 10% of ALS cases are familial (2). Twenty percent of familial ALS cases are associated with mutations to SOD (2). The association of SOD, a free radical scavenging enzyme, with ALS was one of the first findings to reveal the important role that free radicals play in physiology, implying aberrant handlings of free radicals may induce aggressive diseases, such as ALS. New biochemical techniques that have emerged over the past two decades have revealed numerous other roles for free radicals throughout biology.

The recognition of free radicals in biology emerged in the late 1960's when Irwin Fridovich and Joe McCord discovered the enzyme SOD (3). This was the first inference of a biological role for the free radical, superoxide  $(O_2^{-})$ . Superoxide is simply formed by reducing molecular oxygen by one electron. In the mid 1980's the vasodilator, EDRF, or endothelium derived relaxation factor, was discovered to be the small free radical, nitric oxide (4). Since this time free radicals have been detected throughout biology implicating a role, for example, in cardiovascular diseases, diabetes, and neurodegeneration. However the implications and assigned roles for free radicals are only as

reliable as the methods that are used to detect them. What are the roles of free radicals? Which are generated in pathology and what roles do they play in disease? Accurate answers to these questions will depend upon reliable methods to detect free radicals.

In 1990, Beckman et al. proposed that the deleterious actions of the two free radicals, nitric oxide and superoxide, were mediated through forming peroxynitrite (ONOO<sup>-</sup>) via a diffusion-limited, radical-radical combination (5). Although nitric oxide (NO) is commonly regarded as being highly reactive, NO reacts rapidly with a surprisingly small number of biological molecules (6). Hence free radical damage associated with NO is not likely mediated through direct attack, as in the case of the more promiscuous hydroxyl radical, but through an alternative pathway (6). Nitric oxide will react with superoxide essentially every time they collide with the consequent generation of peroxynitrite. Peroxynitrite is a much more potent oxidant than either of its precursors, able to oxidize nucleic acids, proteins and lipids (Fig. 1.1, below) (7). Peroxynitrite anion is capable of oxidizing metal centers (7). Peroxynitrite can be protonated to generate peroxynitrous acid which rapidly rearranges and ~70% decomposes to nitrate and ~30% decomposes into reactive species capable of further oxidative attack, such as the nitration of tyrosine residues (5, 8, 9). The reactive intermediates from peroxynitrous



Figure 1.1 The reactive chemistry of peroxynitrite.

acid have been proposed to be hydroxyl and nitrogen dioxide radicals, although there is some debate as to whether the actual nitrating species is the radical pair or another reactive intermediate (10, 11). Similarly, peroxynitrite can react with carbon dioxide, generating nitrosoperoxycarbonate, which also decomposes to a reactive species (presumably nitrogen dioxide and







Figure 1.2 The spinal cord from a rat expressing the ALS-asscoiated, SOD<sup>G93A</sup> mutation displays increased nitrotyrosine immunoreactivity (red) in the ventral region of the lumbar spinal cord (unpublished observations). carbonate radical) which possesses enhanced tyrosine nitrating capabilities (8). Chapter 2 describes a method to synthesize peroxynitrite and reviews its chemistry. Peroxynitrite formation *in vivo* has been demonstrated through detection of its oxidized substrates, namely nitrotyrosine.

Antibodies to nitrotyrosine have implicated peroxynitrite formation in a number of neurodegenerative disorders including, Alzheimer's, Parkinson's, Hunington's disease and amyotrophic lateral sclerosis (ALS) ALS (12). is а neurodegenerative disease involving progressive paralysis due to the selective loss of motor neurons. Interestingly, tyrosine nitration is one of the earliest markers in degenerating motor neurons of ALS (Fig. 1.2) (12). In 1995 Abe et al. reported that motor neurons within

spinal cords from ALS patients have increased nitrotyrosine immunoreactivity

(13). Levels of free nitrotyrosine are also increased in the lumbar and thoracic spinal cord of ALS patients (14) and in a mouse model of ALS (15). Mouse models of ALS also display increased nitration of proteins from spinal cord homogenates (16, 17). The abundance of nitrotyrosine in ALS models implies peroxynitrite formation and subsequent nitration of tyrosine as a part of ALS pathogenesis. Therefore, inhibiting the nitration of tyrosine by peroxynitrite may be efficacious in inhibiting ALS disease pathogenesis.

The low molecular weight anti-oxidant, urate, is an efficient inhibitor of peroxynitrite-mediated nitration of tyrosine (18). Urate does not directly 'scavenge' peroxynitrite itself, but reacts with the radical intermediates responsible for tyrosine nitration (19). Urate can therefore be used to parse the roles of peroxynitrite and determine if peroxynitrite is acting through radical oxidation and subsequent nitration. Characterization of the reaction of the peroxynitrite-mediated oxidation of urate is presented in Chapter 3. We identified one product, triuret, which yielded the same fragment ions as the product of peroxynitrite-mediated oxidation of urate, as determined by mass spectrometry. The mechanism we proposed depicts the multi-reaction steps involved to generate triuret and how other products might be formed, such as the amino carbonyl radical which has been detected by radical spin trapping (19). The mechanism by which peroxynitrite reacts with urate helps to explain urate's potential as an antioxidant.

Urate has been shown to inhibit peroxynitrite oxidation of proteins (18). In addition, as an antioxidant, urate has been shown to be an efficient scavenger of peroxyl radicals (20, 21), of nitrogen dioxide (22) and an efficient inhibitor of lipid peroxidation (23). Administration of urate significantly reduces damage in ischemic reperfusion injury (24). Urate is also protective against glutamate-induced excitotoxicity, increasing the survival of hippocampal cultures, attenuating the production of ROS from the mitochondria, and maintaining calcium homeostasis (24).

Because of urate's antioxidant potential and the implication of peroxynitrite formation in ALS, we used urate to treat mice over-expressing the ALS-associated mutation G93A to superoxide dismutase (SOD<sup>G93A</sup>). Over-expression of mutant SOD in mice results in motor neuron disease with pathological features similar to those of human ALS (25), and SOD<sup>G93A</sup> over-expressing mice are the current model used to study ALS (2). SOD<sup>G93A</sup> mice were orally administered a diet supplemented with 3% uric acid and 2% oxonic acid. Oxonic acid was added to inhibit uricase, which rapidly degrades uric acid in rodents. Administration of the diet did not alter weight gain and raised plasma uric acid levels approximately two fold (Appendix 1). Uric acid/oxonic acid supplementation beginning at eight weeks of age failed to delay muscle weakness or increase lifespan in SOD<sup>G93A</sup> mice (Appendix 1).

It appeared we were not able to maintain sufficient concentrations in the spinal cord over the two-month duration to be effective in the SOD<sup>G93A</sup> mice. In contrast, in a mouse model of multiple sclerosis the administration of urate decreased nitrotyrosine in addition to inhibiting paralysis and tissue damage (26). To obtain these results, mice were intraperitoneally injected with urate four times daily to increase serum urate five fold (27). Similar administration of injecting higher doses, in contrast to those obtained by oral administration, may be required to inhibit tyrosine nitration and paralysis in SOD<sup>G93A</sup> ALS mice. Our in vitro studies provide important evidence that urate protects against peroxynitrite radical formation and the induction of reactive astrocytes and motor neuron death (28, 29).

The downstream marker of peroxynitrite, nitrotyrosine, has been detected in ALS (12, 14, 16, 30) suggesting peroxynitrite formation. Are the reactant precursors of peroxynitrite, nitric oxide and superoxide, increased in ALS? Several reports indicate increased nitric oxide production in ALS (31). Human spinal cords from ALS patients show increased immunoreactivity for the inducible and neuronal forms of nitric oxide synthase (NOS) in neurons and astrocytes, as compared to controls (32-34). Similarly, transgenic mice

over-expressing SOD<sup>G93A</sup> have increased inducible NOS immunoreactivity in the spinal cord during early symptomatic and end stages of the disease process (35). Therefore we wished to determine if superoxide generation is also increased in ALS.

While the generation of superoxide is not necessarily detrimental, and superoxide often times acts as a reductant instead of an oxidizing species, superoxide and reactive oxygen species (ROS) derived from superoxide have been implicated in a number of pathologies, including neurodegenerative disorders (36-40), aging (36-38), cancer (37), and diabetes (41). There are a number of sources for superoxide production, as follows (42): Superoxide can be generated enzymatically by NADPH oxidase or xanthine oxidase. Although xanthine oxidation largely occurs via xanthine dehydrogenase, which utilizes a transfer of electrons onto NAD<sup>+</sup> instead of oxygen, xanthine dehydrogenase can be converted to xanthine oxidase in injured tissues. Nitric oxide synthase can also catalyze the formation of superoxide especially under conditions of low tetrahydropteridines. Transition metals can catalyze the autooxidation of biological molecules in the presence of oxygen to generate superoxide. These substrates include glyceraldehyde, FMNH<sub>2</sub>, FADH<sub>2</sub>, adrenalin, noradrenalin, L-DOPA, dopamine and some thiol compounds such as cysteine. Transition metals, such as the iron in heme proteins, can also catalyze the formation of superoxide from oxygen. The endoplasmic reticulum has been shown to generate superoxide through a proposed oxidase activity of the cytochrome P450 systems. The nucleus also is a culprit in superoxide generation, having its own membrane electron transport chain which has been shown to generate superoxide. Superoxide can also be generated by activated phagocytic cells. However the main culprit for superoxide production is the mitochondria.

Approximately 85-95% of the oxygen utilized by mammalian cells is consumed by the mitochondria for respiration (37, 43). Oxygen is the ultimate reduction site for electrons flowing through the respiratory chain during oxidative phosphorylation (Figure 3). During the shuffle of electrons, approximately 1-4% of electrons have been estimated to leak and reduce oxygen to superoxide (38, 43, 44). The sites in mitochondria where oxidative damage and increased  $O_2^{\bullet}$  production occur are principally Complexes I and III (Fig. 1.3) (38, 41, 44-47). Complex I, the NADH-ubiquinone oxidoreductase consists of 43 subunits, at least 7 iron sulfur clusters (Fe-S) and two sites that bind coenzyme Q (44, 48). The exact site of  $O_2^{\bullet}$  generation within Complex



Figure 1.3 Possible sites of superoxide generation from the electron transport chain of the mitochondrial inner membrane.

I is controversial (38, 44, 45, 49). Some evidence shows that ubisemiquinone associated with the N2 Fe-S centers can facilitate the transfer of an electron to oxygen to generate  $O_2^{-}$  (44, 49). However, a mutant of Complex I lacking the N2 Fe-S cluster showed the same rate of  $O_2^{-}$  production (45) and an alternative site of  $O_2^{-}$  generation involving the FMNH<sub>2</sub> or FMN semiquinone has been proposed (45, 50). Complex III, the ubiquinol-cytochrome c oxidoreductase, facilitates the transfer of electrons from reduced, two electron carriers, the quinones, to one electron carriers, the cytochromes. Antimycin A increases  $O_2^{-}$  generation from Complex III by blocking electron passage from a seminquinone to cytochomre  $b_H$  (38). Other, unidentified enzymes within the mitochondria could also contribute to  $O_2^{-}$  production and it has been

suggested that the assumption that only Complexes I and III are involved in  $O_2^{-}$  production be reassessed (41). Both cytochrome *c* and complex IV have reduction potentials that are too high to reduce oxygen to superoxide, but there are multiple other complexes that reduce ubiquinone and are potential sources of superoxide. The state of respiration and reduction of respiratory chain carriers also affect  $O_2^{-}$  production (46, 47, 51). During respiration, depletion of ADP or decreased ATP production (State IV) increases the mitochondrial membrane potential, increases reduced electron carriers and consequently increases ROS production (41, 51). The addition of uncouplers to decrease the membrane potential slows the production of ROS in State IV (51).

Oxidant production is balanced by antioxidant defenses such as catalase and glutathione peroxidase which are scavengers of hydrogen peroxide, and glutathione reductase, which maintains a large pool of reduced glutathione (41). MnSOD and cytochrome *c* rapidly scavenge superoxide and these species are also largely responsible for the difficulty of experimentally detecting superoxide.

Assessing the generation of O<sub>2</sub><sup>--</sup> in the mitochondria has been difficult and controversial because the assays currently available to detect superoxide are susceptible to a number of confounding variables (52). In order to detect superoxide, the sensor must be able to compete with intracellular SOD, which is consuming superoxide with a rate constant of approximately 10<sup>9</sup>M<sup>-1</sup>s<sup>-1</sup>. Moreover, the sensor would need to be selective for superoxide over other oxidants. Fluorescent probes to detect oxidants, such as the reduced forms of rhodamine, fluorescein, and ethidium are susceptible to oxidation by intracellular peroxidases and oxidoreductases, and to photooxidation (53-57). Non-specific oxidation can generate their two-electron fluorescent products by processes independent of superoxide oxidation, and are non-selective assays.

The DNA binding fluorescent molecule ethidium can be chemically reduced to hydroethidine (HE), which is perhaps the most common fluorescent

probe used for superoxide detection. Ethidium (Et<sup>+</sup>, 3,8-diamino-5-ethyl-6phenylphenanthridinium) intercalates into nucleic acids to emit red fluorescence (Fig. 1.4). Et<sup>+</sup> can be reduced by sodium borohydride to produce hydroethidine (HE) which does not intercalate into nucleic acid (58). In 1984, Gallop et al. reported that HE is readily taken up and internalized by live cells (59). HE fluoresces blue in the cytoplasm and its oxidized counterpart, Et<sup>+</sup>, fluoresces red in the nucleus (59). HE was used in 1986 as a vital dye to stain live cells of different tumor lines (58). The differences in red fluorescence between cell lines was attributed to the cell-cycle stage and cell metabolic state which were theoretically affecting the cell's ability to dehydrogenate



Figure 1.4 (A) Cultured cells (BPACs) incubated with HE show ethidium fluorescence in the nucleus (arrows), as well as in the cytosol. The site of HE oxidation is unknown. Ethidium fluoresces once intercalated into nuclear DNA, cytosolic RNA, or, to a smaller extent, mitochondrial DNA (B is a zoom of the box in A; unpublished results kindly provided by M.S. Janes).

HE (58). HE was proposed to be dehydrogenated by a NADP<sup>+</sup> and HE was proposed to be useful to measure dehydrogenase activity (58). In contrast to enzymatic dehydrogenation of HE, Rothe and Valet showed *in vitro* that HE

was oxidized by potassium superoxide to a red fluorescent product (53). Flow cytometry was used in combination with HE to analyze respiratory bursts of phagocytes *in vivo* (53). HE was adopted as a fluorescent probe to detect ROS during respiratory bursts (60-62) and emerged as a probe for the detection of  $O_2^{-}$  (52, 63, 64). However in 1990 Rothe et al. showed that HE could be readily oxidized to emit red fluorescence not only by  $O_2^{-}$  but also by  $H_2O_2$  combined with peroxidase (53). HE, dihydrofluorescein and dihydrorhodamine can all be oxidized to their fluorescent counterparts by  $H_2O_2$  with peroxidase, cytochrome c or  $Fe^{2+}$  (53, 54, 56, 65). Dyes such as fluorescein and rhodamine reduced to their non-fluorescent counterparts were called "dihydro" derivatives. Therefore, the reduced derivative of Et<sup>+</sup> became more widely known as dihydroethidium (52, 64, 66), although HE has only one more hydrogen than Et<sup>+</sup> with a second electron placed within the orbital of a nitrogen.

In 1992, HE was used to determine the viability of a variety of bacterial populations and surprisingly, fluorescence was obtained not only from live cells but also from dead bacterial cells (57). HE was suggested to be oxidized by two different processes, a biological and an abiotic process (57). HE was also shown to be oxidized by UV light (57). Swannell et al. proposed HE could be oxidized to more than one oxidation product capable of red fluorescence (57). In 2003, Kalyanaraman et al. reported that HE is oxidized by  $O_2^{-}$  to a product whose fluorescence emission is blue shifted from Et<sup>+</sup> by 30 nm and concluded HE is oxidized by  $O_2^{-}$  to a product that is distinctly different from Et<sup>+</sup> (66) (Scheme 1). Mass spectrometry and NMR were used to ascertain the structure of the  $O_2^{-}$  product of HE to be a hydroxylated product (HO-Et<sup>+</sup>) (67). Detecting HO-Et<sup>+</sup> by HPLC provides a selective technique for  $O_2^{-}$  detection by differentiating and quantitating oxidation of HE to either Et<sup>+</sup> or HO-Et<sup>+</sup> (67). Detecting HO-Et<sup>+</sup> by fluorescence microscopy is difficult because the emission maxima of HO-Et<sup>+</sup> is shifted only ~30 nm from Et<sup>+</sup> (67).

To create a mitochondrially targeted superoxide detector, HE was derivatized with the addition of a hexyl chain to attach a triphenylphosphonium group. This product is commercially available as MitoSOX Red<sup>TM</sup> (Mito-HE; Fig. 1.5, below). The triphenyl phosphonium (TPP<sup>+</sup>) moiety is used to deliver molecules to the mitochondria. Delivery is accomplished by the positive charge being attracted to the negatively charged inner leaflet of the inner mitochondrial membrane established by the membrane potential of the mitochondria. The positive charge on the phosphonium is surrounded by



Figure 1.5 (A) Red fluorescence emitting from the mitochondria in cultured cells (BPACs) incubated with Mito-HE. Red fluorescence is <sup>+</sup> reticular and mitochondrial (B zoom of A, arrows) as compared to BPACs incubated with HE (Fig. 1.4). (C) Structure of Mito-HE.



three lipophilic phenyl groups facilitating transfer across phospholipid bilayers. A similar moiety, the dibenzylammonium cation, was first used in 1969 by Vladimir Skulachev to provide some of the first evidence that mitochondria have a membrane potential ( $\Delta \psi$ ) across the inner membrane (68). This was accomplished by showing the dibenzylammonium cation accumulated within the mitochondria in response to an increase in  $\Delta \psi$  (68). Skulachev later used the methyl TPP<sup>+</sup> cation, which accumulated to a greater extent in energized mitochondria than the dibenzylammonium cation (69, 70). The TPP<sup>+</sup> moiety is commonly used to measure membrane potential (71). More recently the TPP<sup>+</sup> moiety has also been applied in a new, therapeutic role to deliver treatment molecules to the mitochondria (41, 72). In the case of Mito-HE, the TPP<sup>+</sup> moiety is being used to attract a cellular probe to the mitochondria to measure oxidants produced there. Mito-HE is oxidized by superoxide in a manner similar to HE, where non-specific oxidation generates the two electron derivative of ethidium (Mito-Et<sup>+</sup>) and oxidation by superoxide generates the specific, hydroxylated product (HO-Mito-Et<sup>+</sup>), as determined by mass spectrometry and NMR (Chapter 4).

While investigating the oxidation of Mito-HE by superoxide, we discovered the superoxide-specific product, HO-Mito-Et<sup>+</sup>, has a selective excitation wavelength at 396 nm that is not present for Mito-Et<sup>+</sup> (Chapter 4). Therefore, excitation at 396 nm can greatly increase selectivity as compared to the currently accepted practice of exciting at 500 nm, which induces fluorescent emission from both the superoxide and non-specific oxidation product. Technical details of how to detect mitochondrial superoxide generation in isolated mitochondria and cultured cells using this method is described in Chapter 5. In Chapter 6, the Mito-HE method we developed was used to investigate if superoxide generation is increased in astrocytes expressing the ALS associated mutation, SOD<sup>G93A</sup>.

The central nervous system (CNS) contains neurons and three types of glial cells: oligodendrocytes, microglial cells and astrocytes. Oligodendrocytes are responsible for myelination and metabolic support of axons. Microglia initiate immune responses and defense in the CNS. Astrocytes, our particular cell of interest, play a central role in the CNS.

Previously, CNS functionality was thought to be accomplished by neurons specifically and glia were viewed simply as the extracellular binding element in the CNS ("nerve cement") (73). However, it is clear now that astrocytes play an integral role in the birth, life and death of neurons (73). Astrocytes make up a syncytium network connected by gap junctions composed of connexins (73). Connexins are channels composed of 6 symmetrical subunits which form a pore that allows molecules  $\leq$  1 kD to diffuse from cytoplasm to cytoplasm (73). This unique infrastructure allows propagation of signaling waves through the astroglial network providing communication and integration of information rapidly over hundreds of microns (73). Astrocytes are also capable of exocytotic release of secretory vesicles containing neurotransmitters. In this manner astrocytes participate in modulating neuronal activity and synaptic transmission. Endfeet of astrocytes connect to blood vessels providing a link between blood capillaries and neurons (Fig. 1.6, below). The last decade of research has changed our perception of astrocytes as being a "supporting" cell of neurons, to a central component carrying out CNS functions (73).



Figure 1.6 Astrocytes provide neurons with trophic support and connections to blood vessels.

Therefore, although ALS is known as a disease involving the death of motor neurons, astrocytes are also intricately involved in disease propagation (74-76). For example, over-expression of the ALS-

associated mutation, SOD1<sup>G37R</sup>, in mice results in an aggressive form of ALS with extensive reactive

astrocytosis. However, selective expression of SOD1<sup>G37R</sup> in neurons only is not sufficient to induce motor neuron disease (75). In chimeric mice, knocking down the expression of SOD<sup>G93A</sup> in glial cells slows disease progression (76). We therefore investigated what aberrant effects the SOD<sup>G93A</sup> mutation has upon astrocytes and their mitochondria, and if SOD<sup>G93A</sup> expression increases

mitochondrial superoxide production. SOD<sup>G93A</sup> has been reported to associate with spinal cord mitochondria (77). Moreover, mutant SOD expression causes mitochondrial morphological alterations (78) and a shift in mitochondrial redox state (79). Indeed, analysis of astrocytes over-expressing SOD<sup>G93A</sup> revealed an increase in mitochondrial superoxide generation (Chapter 6). Interestingly, fluorescence microscopy also revealed intense staining of SOD<sup>G93A</sup> colocalizing with the mitochondria, supporting the probability of detrimental effects of SOD<sup>G93A</sup> association with mitochondria.

These investigations may contribute to determining the mechanisms of disease progression mediated by mutant SOD in familial ALS, supporting a role for mitochondrial dysfunction in neurodegeneration. The causes of Alzheimer's disease, Parkinson's disease, Huntington's disease, and ALS are unknown, however each demonstrates the progressive loss of neuronal Although each neurodegenerative disorder is distinct in populations. character, some overlap can be observed in the Guam cases of Parkinsonism-Dementia-ALS complex which demonstrate pathological features in combination (6). Mitochondrial dysfunction may be a common underlying mechanism shared in neurodegenerative pathways. This work provides methods of increased quality to assess mitochondrial dysfunction and free radical generation of superoxide. These methods can be utilized to investigate not only neurodegenerative disorders, but also other pathologies in which free radical formation is implicated.

Chapter 2

Synthesis of Peroxynitrite from Nitrite and Hydrogen Peroxide.

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We describe here a simple synthesis of peroxynitrite (ONOO<sup>-</sup>) from nitrite and hydrogen peroxide for biochemical studies which can be safely and rapidly conducted using readily available equipment in any laboratory. Residue nitrite and hydrogen peroxide, the two most troublesome contaminants, are less than 2% of the peroxynitrite concentration. Many syntheses for peroxynitrite have been reported, but are potentially hazardous and require expensive, specialized equipment. The cleanest method produces solid tetramethylammonium peroxynitrite without contaminants (80). However, this method requires grinding potassium superoxide at -77°C in highly toxic liquid ammonia in a potentially explosive reaction. Several fume hoods have been destroyed during the synthesis of the intermediate tetramethylammonium superoxide. Solid peroxynitrite is commercially available but is expensive even for small amounts. Significant contamination with nitrite can result even when pure peroxynitrite is dissolved in alkaline solutions unless considerable care is taken (81). Alternatively, peroxynitrite may be synthesized by bubbling ozone through alkaline solutions of azide (82). Ozone needs to be generated by an ozone generator from oxygen and ozone is an extremely strong pulmonary and eye irritant. Residual sodium azide is toxic and a significant confounding factor in biological experiments using peroxynitrite. If the azide reaction is run to completion, significant contamination with nitrite occurs. It is also possible to synthesize peroxynitrite by crushing solid potassium superoxide with sand in an anerobic system and then flushing with nitric oxide gas (83). This method requires substantial plumbing with stainless steel tubing to handle nitric oxide, significant time and yields relatively small quantities of peroxynitrite. The peroxynitrite is contaminated with a large amount of hydrogen peroxide formed by the dismutation of unreacted superoxide.

A simpler method of synthesis was first performed in 1902 when Baeyer and Villiger (84) published a method of mixing nitrite with hydrogen peroxide. In 1968, Hughes and Nicklin (85-88), reported a synthesis also based upon the reaction between nitrite and hydrogen peroxide in which the hydrogen peroxide was 'thrown' into a nitrite solution and produced peroxynitrite in yields between 40 to 50%. In 1973, Reed and coworkers (89) reported a method reacting nitrite and hydrogen peroxide in a quenched flow reactor. This apparatus was made up of a Lucite rod bored for the inlet of reactants. Yields increased to approximately 82%. Beckman et al. (90) also previously published a method of peroxynitrite synthesis which utilized a vacuum system to pull reactants through the mixing chamber. This method had variable yields depending upon the time of day. Yields were highest when syntheses were performed at night because the house vacuum had a stronger pull. Saha et al. (91) also searched for optimal conditions with fewer contaminants and published a method in which they constructed a rapid mixer commonly used in stopped flow machines. This required fine machining of two specialized small rapid mixing chambers.

We herein report a simpler version of the Saha et al. method that requires only a syringe pump, four tee-connectors and flexible tubing which optimizes synthesis conditions. The method can produce hundreds of milliliters of ~180 mM peroxynitrite in less than an hour. It can be scaled down to make small quantities of isotope-labeled peroxynitrite. An improved procedure for making manganese dioxide is also described to better remove residual hydrogen peroxide. The major contaminant in the peroxynitrite synthesized by this method is nitrate, which generally has little influence on the use of peroxynitrite for most experiments.

#### The chemistry of the reaction.

Peroxynitrite is synthesized by reacting acidified hydrogen peroxide with sodium nitrite. The pKa of nitrite is ~3.4. Under moderately acidic conditions nitrous acid is an efficient nitrosonium donor. The nucleophilic attack upon hydrogen peroxide produces peroxynitrous acid:



Under acidic pH peroxynitrous acid has a half life of less than one second. But the reaction can be quickly quenched with an excess of base to yield peroxynitrite anion in high concentrations.

#### Synthesis of peroxynitrite.

Typically, three solutions are freshly prepared in high-quality water: 0.7 M HCl + 0.6 M H<sub>2</sub>O<sub>2</sub> (14.5 ml of conc. HCl + 17.0 ml of 30% H<sub>2</sub>O<sub>2</sub> into a final volume of 250 ml), 0.6 M sodium nitrite (10.4g into 250 ml of water); and 3 M sodium hydroxide (30g into 250 ml of  $H_2O$ ). Yields are highest when all solutions are at room temperature. The use of higher concentrations of reactants can result in the hazardous generation of toxic gases that can blow off the tubing. Goggles should be worn during the synthesis for the possibility that tubing disconnects. High quality sodium nitrite should be freshly purchased and reserved for the synthesis. Five 30 ml disposable plastic syringes (Becton Dickinson) are needed. Two are filled with sodium nitrite, two filled with the acidified hydrogen peroxide, and the last syringe is filled with 3 M sodium hydroxide. Tubing should be connected to syringes using plastic Luer lock connectors and not by using steel needles. The tubing is connected using plastic T's as shown in Figure 2.1. The nitrite and peroxide reactants are flowed through tygon tubing and Teflon tubing was used after the first T junction for the peroxynitrite product flow.



Figure 2.1 0.7 M HCl + 0.6M  $H_2O_2$  and 0.6 M NaNO<sub>2</sub> are loaded into syringes as in the diagram. The reaction is quenched with 3 M NaOH in the syringe downstream. The lengths of the tubing have been optimized for the highest yields. Metals are avoided in any connections. All five syringes are loaded side by side onto a syringe pump which is ran at a maximal flow of 17ml/min.

The indicated lengths of tubing have been varied to optimize yields. The tubing is 3 mm inside diameter, except for the tube measuring 5.8 cm, which is ~ 2 mm in diameter. Any metal should be avoided as described in this method, such as the use of metal syringe tips, T connections, and tubing. Trace contamination with transition metals catalyzes the alkaline decomposition of peroxynitrite to nitrite and dioxygen (92). The principal advantage of this arrangement is to have acidified nitrite and hydrogen peroxide to mix in the first set of tees and then to be further mixed at the second tee junction.

The five syringes are loaded side by side onto a syringe pump. We successfully secured five 30 ml syringes on a Harvard Syringe Pump 22 with laboratory tape, however syringe pumps are commercially available that will hold five syringes. The syringe pump is run at its maximum rate (17 ml/min

using 30 ml disposable syringes). The flow is diverted to waste until a yellow solution emerges. The yellow peroxynitrite solution is then collected on ice in a separate flask. The concentration of peroxynitrite ranges from 180-190 mM. The concentration of peroxynitrite can be easily measured spectrally at 302 nM. For absorption measurements, the stock solution needs to be diluted 2 µL into 1000 µL of 100 mM NaOH. It is necessary to repeatedly invert the cuvette to ensure adequate mixing. Repeating the dilution and absorption recommended for accurately measurements are determining the concentration. The first reported extinction coefficient was estimated to be 1670 M<sup>-1</sup>cm<sup>-1</sup> and is the most likely used value at present. However, the extinction coefficient has been more accurately determined using pure tetramethylammonium peroxynitrite to be 1700 M<sup>-1</sup> cm<sup>-1</sup> (80).

The synthesis of peroxynitrite gives an approximate 70% yield. The remainder is mostly nitrate. It should be noted that the solution contains approximately 0.28 M NaCl as well 0.1 M NaOH. If it is desired to avoid NaCl, 0.7 M nitric acid may be used in place of HCl in the starting hydrogen peroxide solution (93).

Approximately one hundred milliliters of the fresh peroxynitrite solution is transferred to a 250 ml beaker whose inner flat surface is thinly covered in manganese dioxide flakes (whose synthesis is described below). The beaker is submerged in an ice bath and the mixture is left to react on ice for 15 minutes without any stirring. This length of time is adequate to consume any unreacted hydrogen peroxide. The peroxynitrite can then be cleaned from manganese dioxide by vacuum filtration using a #2 paper filter. Residual  $H_2O_2$ is assayed as described below. Solutions of peroxynitrite are then frozen in 1 ml aliquots at -80°C for up to one year. We no longer attempt to freezefractionate peroxynitrite because this led to a large increase in nitrite contamination relative the increase in peroxynitrite concentration (5).

We were concerned that plasticizers might leach from the tubing into the reactants during the synthesis process. When the synthesized peroxynitrite was analyzed by electrospray mass spectrometry in positive ion mode, a number of unidentifiable peaks appeared after treatment with commercially available  $MnO_2$  but not before treatment. These peaks were not present when  $MnO_2$  was synthesized, which is easily done as described below. This  $MnO_2$  was also far more effective at catalyzing hydrogen peroxide decomposition.

### Manganese dioxide synthesis.

Residual hydrogen peroxide is eliminated by cleaning with manganese dioxide ( $MnO_2$ ), which catalytically decomposes hydrogen peroxide. To prepare  $MnO_2$ , 8 g of potassium permanganate (caustic) is dissolved in 50 ml water and 500 ml of 95% ethanol is slowly added. The reaction is stirred overnight. The dark brown precipitate is collected the next day by vacuum filtration and washed with 2-3 liters of water. It is allowed to dry completely for several days. Yields are approximately ~97%. The brown product is broken up into small, ~5 mm flakes. Excessively crushing the  $MnO_2$  should be avoided because as the  $MnO_2$  becomes increasingly powdery it becomes difficult to filter the  $MnO_2$  out of the peroxynitrite. Once the  $MnO_2$  has been filtered out of the peroxynitrite solution, it can be left to dry and used again. However, once the  $MnO_2$  becomes too powdery it is discarded.

Hydrogen peroxide concentrations are determined using the horseradish peroxidase (HRP) based assay, using 4 mM 3,5-dichloro-2-hydroxybenzene sulfonic acid (sodium salt) and 3 mM 4-aminoantipyrine, and 0.01 unit/ml HRP in a 1ml final volume assay. When performing the HRP assay, peroxynitrite solutions should be first decomposed by diluting peroxynitrite into buffer (100 mM potassium phosphate, 40  $\mu$ M DTPA, pH 7.3) and then incubated at 37°C for 15 minutes. The absorbance is monitored at 510 nM and a standard curve should be prepared from stock H<sub>2</sub>O<sub>2</sub> solutions.

Peroxynitrite interferes with the peroxidase assay and therefore needs to be decomposed first. Caution needs to be taken to ensure the peroxynitrite is diluted adequately because excess hydrogen peroxide will lead to inaccurate results. For example, before the peroxynitrite is cleaned with  $MnO_2$  it is necessary to dilute the peroxynitrite into buffer by 1/2000 using a series of dilutions (first  $10\mu$ L ONOO<sup>-</sup> into  $100 \mu$ L buffer, then 5  $\mu$ L into the 1 ml assay). Once the peroxynitrite has been cleaned with  $MnO_2$  a dilution factor of 1/20 is adequate. In this case 50  $\mu$ L of peroxynitrite is added to 1 ml buffer and then the aminoantipyrine and 3,5-dichloro-2-hydroxybenzene sulfonic acid reactants can be added after the peroxynitrite has been decomposed.

Before cleaning with MnO<sub>2</sub> the fresh peroxynitrite contained ~16 mM  $H_2O_2$ . After cleaning peroxynitrite for 15 minutes with manganese dioxide as described above, hydrogen peroxide levels decreased to ~80 µM. The lower limit of detection in stock solutions was approximately 50 µM  $H_2O_2$ . After synthesis the peroxynitrite concentration of peroxynitrite ranges from 160-180 mM and decreases to 140-160 mM after MnO<sub>2</sub> cleaning. Using an excess of 0.7 M  $H_2O_2$  in the synthesis can increase yields to 170-190 mM peroxynitrite. However this increases the  $H_2O_2$  contamination to 50 mM before MnO<sub>2</sub> treatment. This excess  $H_2O_2$  contamination is only decreased to ~10 mM by treating with MnO<sub>2</sub> for 15 minutes. Therefore it is not adventitious to use the excess  $H_2O_2$  in the peroxynitrite synthesis.

#### Working with peroxynitrite.

It is essential to recognize that several contaminants are present in high concentrations in the final solution of peroxynitrite. These are principally sodium chloride, nitrite, nitrate, oxygen and sodium hydroxide. By using the Griess method, we find levels of nitrite to be ~3.5 mM in 180 mM peroxynitrite solutions. Latal et al. (81) recently reported a larger contamination (20mM) of nitrite can result from thawing peroxynitrite at warmer temperatures. We did not observe a similar build up of nitrite when thawing 1 ml aliquots at 0°C, 25°C or 37°C. Possibly, the increase in nitrite contamination from thawing at 37°C may be specific to the dissolution of the tetramethylammonium salt or affected by differences in salt or alkali concentrations.
peroxynitrite solutions on ice is generally preferable because it reduces the decomposition of peroxynitrite that occurs at warmer temperatures.

Nitrite may also accumulate as peroxynitrite is stored on ice and is accompanied by the formation of oxygen bubbles along the tube walls of peroxynitrite solutions as they are thawed. It is important to perform a control with the reverse order of addition to allow peroxynitrite to decay. This control experiment will help reveal effects due to the contaminants rather than from peroxynitrite itself. The present synthesis is a simple means to prepare either very small or large amounts of peroxynitrite, which is suitable for many biological experiments. However, caution is needed to be sure that appropriate controls are made to control for residual contaminants. Nitrate and nitrite contaminants are also produced during the decomposition of pure peroxynitrite. Other considerations about working with stock solutions of peroxynitrite have been previously described (94).

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Chapter 3

Triuret: a novel product of peroxynitrite-mediated oxidation of urate.

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## 3.1 Abstract

Urate is an efficient antioxidant and has recently emerged as a competitive inhibitor of tyrosine nitration by peroxynitrite. In vivo and in vitro studies demonstrate the large extent to which urate prevents nitration and establish the biological importance of the reaction between urate and peroxynitrite. The existing lack of characterization of this reaction has led us to focus our studies upon the mechanism of urate oxidation and the products formed. An oxidation product has been previously isolated and mass spectrometry revealed a mass of 146, which spontaneously fragmented into several other ion peaks without use of MS/MS mode. Here, we propose the novel oxidation product to be triuret (H<sub>2</sub>NCONHCONHCONH<sub>2</sub>). Triuret accurately reproduced the peculiar mass spectrum. Identification of the oxidation product helps to develop the mechanism of peroxynitrite-mediated oxidation of urate and can help explain urate's potential as both an antioxidant for tyrosine nitration while paradoxically acting as a pro-oxidant for lipids and sulfhydryls.

#### 3.2 Introduction

Long before peroxynitrite was recognized as a biological oxidant, Bruce Ames et al. (95) proposed that urate could be a major antioxidant in humans. Although primates lost the ability to synthesize ascorbate, a point mutation to uricase resulted in increased serum urate levels by preventing its enzymatic degradation. Ames et al. (95) proposed that urate might be a partial replacement of vitamin C as an antioxidant in humans.

One possible advantage of urate compared to ascorbate is urate's substantially higher reduction potential, making urate less likely to reduce metals which can catalyze Fenton chemistry. Urate is also an effective scavenger of peroxyl radicals (ROO<sup>\*</sup>). More recently, urate has been found to be an effective inhibitor of peroxynitrite-mediated nitration of tyrosine residues. Urate only slightly increases the rate of peroxynitrite decomposition (<30%), which indicates that urate is not directly scavenging either peroxynitrite anion or peroxynitrous acid. Rather, urate is competing for the radical intermediates formed during the decomposition of peroxynitrite, which are responsible for tyrosine nitration. Urate is also effective in reducing tyrosine nitration by peroxynitrite in the presence of carbon dioxide, which is a major reactant for peroxynitrite in vivo.

Urate administration significantly decreased nitrotyrosine formation in brain and greatly reduced disease severity in the experimental autoimmune encephalitis mouse model of multiple sclerosis, (26) even when urate levels were raised after disease symptoms had started. Mice treated with urate prior to onset of the disease had fewer macrophages and other inflammatory cells migrating across the blood–brain barrier (BBB), implying that urate may reduce peroxynitrite-induced permeability of the BBB to inflammatory cells. Several other studies also show the enhanced inhibition of nitration by urate. For example, the endogenous buildup of urate in rodent heart protects proteins from peroxynitrite-mediated protein nitration (18). Urate (100  $\mu$ M) increased survival of neurons after prenatal hypoxic injury in rat embryos from

20 to 80% neuronal survival (24). The involvement of urate in these studies suggests that nitration is a significant contributor in pathology and gives increasing rise to the importance of the oxidation of urate by peroxynitrite and its mechanism of protection. Surprisingly little is known about peroxynitrite-induced urate oxidation mechanisms, in part due to the complexity of multiple reaction pathways that yield a complex series of products.

The oxidation of purine bases by peroxynitrite is well known to increase susceptibility to further oxidation by peroxynitrite to form a complex array of products (96). Thus, secondary reactions of products formed in the oxidation of urate may be important in preventing tyrosine nitration. Oxidized products could potentially be useful as markers of peroxynitrite. Skinner et al. (97) have tentatively identified one such product. When urate was oxidized with a 2-to 3fold excess of peroxynitrite, a novel oxidation product was isolated by HPLC and subsequently subjected to an electrospray mass spectrometer (97). The resulting spectrum revealed a prominent peak (Mr = 146) that was proposed to be 2-nitrito-4-amino-5-hydroxyimidazoline (Fig. 3.1). We were intrigued by this compound because the nitrito (ONO-) moiety could potentially provide a useful biological marker for peroxynitrite and be a source of biologically active nitric oxide. The reported mass spectrum also contained a series of smaller puzzling peaks, suggesting that the parent ion was spontaneously fragmenting in the mass spectrometer. During an ongoing investigation of how urate inhibits tyrosine nitration by peroxynitrite, we found that an alternative oxidation product called triuret of mass 146 might be formed by the oxidative decarboxylation of allantoic acid (see Fig. 3.1). Triuret, or diimidotricarbonic diamide, is a commonly used fertilizer. We report here that triuret produces the same fragmentation pattern in a single quadrupole mass spectrometer as the peroxynitrite-mediated oxidation product of urate, with mass of 146.

## 3.3 Materials and methods

Triuret was obtained from the Sigma–Aldrich library of rare chemicals. A stock solution of 6 mM triuret was prepared with gentle heating in 20 mM NH<sub>4</sub>OH. Ammonium hydroxide was obtained from Fischer and acetonitrile from Burdick and Jackson. Aliquots were diluted with water to 300 IM and loop injections were made directly onto the electrospray interface of a PE-Sciex API III mass spectrometer in positive ion mode. The orifice potential was set to 70 V and the needle to 5000 V. The mobile phase was 50% water and 50% acetonitrile with 8  $\mu$ L/min flow rate.

#### 3.4 Results

Triuret produced a spectrum in single MS mode (Fig. 3.2) with ion peaks m/z at 61, 87, 104, 130, 147, and 169, identical to that reported for the product of urate reacted with an excess of peroxynitrite. Sodium adducts produced a peak of mass 169, which coincidentally was the same mass as urate. Protonation of triuret (m/z = 147) would be expected to favor the spontaneous fragmentation of the weak amide bonds of triuret during electrospray ionization and desolvation (Fig. 3.3). This results in the initial neutral loss of ammonia to yield product I (m/z = 130). Triuret can also fragment to lose isocyanic acid (O = C = NH) to yield biuret (m/z = 104), which can further lose either ammonia (product II, m/z = 87) or an additional isocyanic acid to produce urea (m/z = 61).

#### 3.5 Discussion

The mass spectrum of triuret accurately reproduces the unusual spectrum previously reported from peroxynitrite-induced oxidation of urate (97). Radical attack on triuret could also readily explain the puzzling detection of amino carbonyl radicals trapped as DMPO adducts by Santos et al. (19) and might contribute to some of the pro-oxidant effects reported from urate oxidation (19). However, triuret would not account for the production of nitric oxide previously reported from urate reacted with peroxynitrite (97). However, nitric oxide could have been produced from other products also generated from peroxynitrite reacting with urate. Skinner et al. (97) did not show that their

novel product itself generated nitric oxide, only that a large excess of peroxynitrite reacting with urate could produce nitric oxide over a 2 hr period.

A number of possible mechanistic pathways have been postulated for urate oxidation. Extensive work in NMR has helped determine intermediates of urate oxidation by uricase (98). Urate is most likely oxidized by radical intermediates derived from peroxynitrous acid or the carbon dioxide-adduct with peroxynitrite in two successive one-electron steps, producing a urate radical and then dehydrourate (Fig. 3.4). Dehydrourate is particularly susceptible to nucleophilic additions and reacts rapidly with water to form 5hydroxyisourate, a previously characterized intermediate of the uricase oxidation. Due to the instability of this intermediate, subsequent hydrolysis of 5-hydroxyisourate produces 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) (98). An equilibrium can then be established with the addition of water to form Intermediate A (Fig. 3.4). Loss of carbon dioxide and the addition of water will produce Intermediate B. Intermediate B could also readily lose carbon dioxide and, in the presence of a strong oxidant such as peroxynitrite, become oxidized to triuret. The net result is a four-electron oxidation accompanied by the addition of four waters and loss of two carbon dioxides. We do not propose that this is the precise mechanism by which urate is oxidized by peroxynitrite but rather only one potential pathway. For example, oxidative decarboxylation of the metabolite allantoic acid might also produce triuret.

Another consideration is that some of the proposed intermediates such as dehydrourate should be capable of adding peroxynitrite directly. Reactions of this manner could contribute to the efficient inhibition of nitration by urate and the slight acceleration in the rate of peroxynitrite decomposition observed by Santos et al. (19).

The dark side of urate cannot be ignored when considering the antioxidant properties of urate. Paradoxically, urate has pro-oxidant properties as well. For example, Santos et al. (19) observed an increase in lipid

peroxidation by peroxynitrite in the presence of urate. Animal models with mild hyperuricemia demonstrate an increase in blood pressure, suggesting that urate may contribute to hypertension (99). However, it has been noted by Ames et al. (95) that the human kidney actively reabsorbs >90% of urate from the kidney. This may be an important consideration in the animal model of hyperuricemia, as most causes of hypertension likely stem from renal failure. Lastly, elevating urate levels conjure concerns about gout. However, gout requires years of exposure to millimolar concentrations of urate as well as genetic susceptibility. The concentration required to develop gout is about 10fold higher than that needed to provide substantial antioxidant activity against peroxynitrite-mediated nitration.

Oxidation of urate in humans can be inferred by the presence of allantoin. The urate oxidation product allantoin has no other known enzymatic mechanism of synthesis and has therefore become a marker of oxidative stress. Triuret may also be a useful marker of oxidative stress. Because multiple oxidative mechanisms can produce triuret, it cannot be used as a specific marker of peroxynitrite. Assay of triuret from patients will also be more difficult than allantoin, due to the reduced stability of triuret to hydrolysis.

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Fig. 3.1 Potential products of peroxynitrite-mediated oxidation of urate.



Fig. 3.2 Mass spectrum of triuret (diimidotricarbonic diamide). The parent compound, triuret, spontaneously fragments after ionization in the mass spectrometer to reveal several additional ion peaks which are consistent with fragmentation of the weak amide bonds and subsequent neutral loss of ammonia, urea or isocyanic acid.



Fig. 3.3 Fragmentation pattern of triuret. Protonation of N1/N4 results in the neutral loss of ammonia and Product I (m/z = 147). Protonation of N2/N3 would be followed by the rapid loss of isocyanic acid and produce biuret (m/z = 104). Protonation of biuret will similarly result in the loss of ammonia (to produce Product II, m/z = 87) or the loss of isocyanic acid to produce urea.



Fig. 3.4 Pathway for urate oxidation. Urate is oxidized to the urate radical and then to dehydrourate. Dehydrourate is susceptible to nucleophilic additions

and reacts rapidly with water to form 5-hydroxyisourate. Subsequent hydrolysis produces 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) which equilibrates with water. Loss of carbon dioxide to produce Intermediate A is followed by the addition of water to produce Intermediate B. Intermediate B will also readily lose carbon dioxide and in the presence of a strong oxidant, such as peroxynitrite, be oxidized to triuret.

Chapter 4

# Selective fluorescent imaging of superoxide in vivo using ethidium based probes.

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#### 4.1 Abstract

The putative oxidation of hydroethidine (HE) has become a widely used fluorescent assay for the detection of superoxide in cultured cells. By covalently joining HE to a hexyl triphenylphosphonium cation (Mito-HE), the HE moiety can be targeted to mitochondria. However, the specificity of HE and Mito-HE for superoxide in vivo is limited by autooxidation as well as by nonsuperoxide dependent cellular processes that can oxidize hydroethidine probes to ethidium. Recently, superoxide was shown to react with HE to generate 2-hydroxyethidium (Zhao, 2003, Free Radic Biol Med, 34, 1359). However, 2-hydroxyethidium is difficult to distinguish from ethidium by conventional fluorescence techniques exciting at 510 nm. While investigating the oxidation of Mito-HE by superoxide, we found that the superoxide product of both HE and Mito-HE could be selectively excited at 396 nm with minimal interference from other non-specific oxidation products. The oxidation of Mito-HE monitored at 396 nm by antimycin-stimulated mitochondria was 30% slower than at 510 nm, indicating that superoxide production may be overestimated at 510 nm by even a traditional superoxide-stimulating, mitochondrial inhibitor. The rate-limiting step for oxidation by superoxide was 4  $x \ 10^{6} \ M^{-1} s^{-1}$ , which is proposed to involve the formation of a radical from Mito-HE. The rapid reaction with a second superoxide anion through radical-radical coupling may explain how Mito-HE and HE can compete for superoxide in vivo with intracellular superoxide dismutases. Monitoring oxidation at both 396 and 510 nm excitation wavelengths can facilitate the more selective detection of superoxide in vivo.

## **4.2 Introduction**

Oxidative stress resulting from mitochondrial dysfunction has been implicated in neurodegeneration, aging, cancer and diabetes(37, 41). Oxidative damage to mitochondrial electron transport complexes and DNA can lead to mitochondrial DNA mutations, aberrant electron transport, disruption of calcium homeostasis and activation of apoptosis (46). Mitochondria consume ~85-95% of the oxygen inspired during respiration (37), most of which is reduced to water, but a small portion (estimates range from less than 0.1 to as high as 4%) of electrons leak from the respiratory chain to reduce oxygen to superoxide ( $O_2^{-}$ ) (44, 100). Complex I and III are major sites of  $O_2^{-}$  formation (44, 46). Both membrane potential and reduction state of respiratory chain carriers affect  $O_2^{-}$  production (46).

Assessing the generation of O<sub>2</sub><sup>-</sup> in mitochondria is confounded by the lack of a sensitive and specific assay (52). Some  $O_2^{-}$  sensors actually generate O<sub>2</sub><sup>-</sup> by either uncoupling the respiratory chain (e.g. luminol) or reacting with oxygen (e.g. luminol and nitroblue tetrazolium) (101, 102). Many O2<sup>•</sup> sensors react with intracellular oxidoreductases and can artificially generate a "superoxide" signal (e.g. hydroethidine, cytochrome c, nitroblue tetrazolium, epinephrine, lucigenin and luminol) (102). To circumvent these technical problems, we report a novel technique to detect O2<sup>-</sup> using MitoSOX  $\operatorname{Red}^{\mathsf{TM}}$ . hydroethidine and the recently developed probe. mitochondrial superoxide indicator (Invitrogen Corp; Mito-HE). Mito-HE comprises hydroethidine (HE), a commonly used probe for O2, inked via a hexyl carbon chain to a triphenylphosphonium  $(TPP^{+})$  group.  $TPP^{+}$  cations target molecules to mitochondria because the positive charge on the phosphonium is surrounded by three lipophilic phenyl groups which facilitates movement across phospholipid bilayers and accumulation into the mitochondrial matrix in response to the negative membrane potential (71).

Hydroethidine is the two-electron reduced form of ethidium (Et<sup>+</sup>, 3,8diamino-5-ethyl-6-phenylphenanthridinium). In 1984, Gallop *et al.* reported that HE is readily taken up and internalized by live cells where HE can be oxidized to Et<sup>+</sup> which intercalates into nucleic acid, greatly enhancing its fluorescence when using 535 nm excitation and 610 nm emission wavelengths (58). Oxidation to Et<sup>+</sup> was originally attributed to the metabolic state of the cell and the cell's ability to dehydrogenate HE (58). However, in 1990, Rothe and Valet showed in vitro that HE was oxidized by potassium superoxide to a red fluorescent product (53). HE has since been widely used to detect reactive oxygen species during the phagocytic respiratory burst (53, 61) and for the detection of intracellular O2<sup>•</sup> (52, 63). However, Rothe et al. also showed that HE was not only oxidized by  $O_2^{\bullet}$  but also by  $H_2O_2$  plus peroxidase (53). HE may also be oxidized by other intracellular processes, involving oxidases or cytochromes, to yield  $Et^+$  (53-56, 103). Consequently, increased Et<sup>+</sup> fluorescence is not necessarily proof of O2<sup>-</sup> production. Swannell et al. proposed HE could be oxidized to more than one red fluorescent product (57). In 2003, Zhao et al. reported that HE is oxidized by O2<sup>--</sup> to yield a hydroxylated product (HO-Et<sup>+</sup>) (66). The initial oxidation of HE had been proposed to involve the formation of a radical (103) implying the oxidation of HE by  $O_2^{-1}$ involves a two step mechanism: (Scheme 1):

HE 
$$\xrightarrow{-1e^-}$$
 HE  $\xrightarrow{-1e^-}$  HO-Et<sup>+</sup>

HO-Et<sup>+</sup> can be separated from Et<sup>+</sup> by HPLC, providing a specific  $O_2^{-}$  assay (66). However, detection of HO-Et<sup>+</sup> by fluorescence microscopy is confounded because its emission spectrum strongly overlaps the emission of Et<sup>+</sup> (66, 67).

We investigated the oxidation of Mito-HE by  $O_2^{-}$  and found Mito-HE was oxidized by  $O_2^{-}$  in a manner similar to HE (Scheme 1). During the course of these investigations, we found that the  $O_2^{-}$  derived product of Mito-HE has a

distinct excitation wavelength at 396 nm that was not present for other oxidation products. HO-Et<sup>+</sup> was also selectively excited at this wavelength. The ability to separate the fluorescence of the  $O_2^{\bullet}$  derived product from other less specific oxidation products can improve the detection and imaging of intracellular  $O_2^{\bullet}$  production.

#### 4.3 Results

#### The selective oxidation of Mito-HE.

Superoxide generated by xanthine oxidase oxidized Mito-HE to give two closely eluting peaks by reverse-phase HPLC (Fig. 4.8). The later peak was determined to be the two electron oxidized form of Mito-HE (Mito-Et<sup>+</sup>) by co-elution with standards and mass spectrometry. The first peak contained one additional oxygen and was identified as the hydroxylated product, HO-Oxidation of Mito-HE by hydrogen peroxide, peroxynitrite, Mito-Et<sup>+</sup>. hypochlorous acid, or a hydroxyl radical generating system generated only a small percent of the fluorescence obtained from oxidation by  $O_2^{-1}$  (Table 4.1). The fluorescence emission maximum for HO-Mito-Et<sup>+</sup> is blue shifted ~20 nm relative to Mito-Et<sup>+</sup> (Fig. 4.1a) similar to what was reported for the HE superoxide product, HO-Et<sup>+</sup> (66). Interestingly, the excitation spectrum of purified HO-Mito-Et<sup>+</sup> revealed an excitation maximum at 396 nm that was not present for Mito-Et<sup>+</sup> (Fig. 4.1b). Excitation at 396 nm (Fig 4.1c) enhanced the fluorescence emission of HO-Mito-Et<sup>+</sup> by 70%, and reduced the spectral overlap of Mito-Et<sup>+</sup> from 40% to 10%. The excitation spectrum of HO-Et<sup>+</sup> also had a distinct excitation at 396 nm that was not present for  $Et^+$  (Fig. 4.9). Therefore excitation at 396 nm could serve as a more selective means for detecting  $O_2^{-1}$  using either HE or Mito-HE.

## Detection of mitochondrial superoxide.

Mito-HE accumulated in isolated mitochondria in a manner dependent on the mitochondrial membrane potential, as well as by adsorption to

membranes (Fig. 4.2). To test the relative ability of Mito-HE to detect  $O_2^{\bullet}$ using  $\lambda_{ex}$ =396 nm, rat heart mitochondria respiring in State III were incubated with Mito-HE (Fig. 4.3). When oxygen concentrations were near the saturation limit for buffer (~230  $\mu$ M), mitochondria oxidized Mito-HE at a rate corresponding to 0.23 nmol  $O_2^{-1} \cdot \min^{-1} \cdot mg$  protein<sup>-1</sup>, while consuming 190 nmol O atm•min<sup>-1</sup> • mg protein<sup>-1</sup>. This corresponds to 0.1% of total electron flux resulting in O2<sup>-</sup> formation. As mitochondria consumed oxygen to concentrations below 100 µM, endogenous superoxide formation was no longer observable. Antimycin stimulated O<sup>--</sup> production by Complex III to 0.81 nmol  $O_2^{\bullet-}$  •min<sup>-1</sup>•mg protein<sup>-1</sup> measured at  $\lambda_{ex}$ =396 nm (Fig 4.3d). The rate of antimycin stimulated fluorescence was 31% faster at  $\lambda_{ex}$ = 510 nm (Fig. 4.3e) as compared to  $\lambda_{ex}$ =396 nm (Fig 4.3d). Because HO-Mito-Et<sup>+</sup> is less fluorescent at  $\lambda_{ex}$ =510 nm, the 31% faster rate indicated that antimycin must have increased the formation of Mito-Et<sup>+</sup> in addition to HO-Mito-Et<sup>+</sup>. The ratio of fluorescence emission from the two excitation wavelengths can be useful to assess nonspecific oxidation versus oxidation mediated by O2<sup>-</sup>.

The two excitation wavelengths can be adapted to more selectively image O<sub>2</sub><sup>--</sup> in living cells. Standard lasers available on confocal microscopes can excite fluorophores at 405 and 514 nm, which are close enough to use in place of 396 and 510 nm. The fluorescence of cultured oligodendrocytes incubated with Mito-HE was more intense using 405 nm excitation, yet qualitatively similar to 514 nm excitation (Fig. 4.4a-d). Fluorescence colocalized with mitochondria, as visualized with MitoTracker<sup>™</sup> Deep Red (Invitrogen Corp, data not shown). Antimycin treatment increased the fluorescence at both excitation wavelengths supporting dependency upon superoxide (Fig 4.4e-h). We have used the two excitations with confocal microscopy to observe increased mitochondrial localized fluorescence in five different types of primary cell cultures with the addition of superoxide-generating agents such as antimycin, paraquat and menadione (not shown).

This method can be adapted for widefield microscopy as well using a custom filter set (Fig. 4.10).

## Mechanism of oxidation.

When Mito-HE was oxidized by xanthine oxidase, the fluorescence at 396/580 nm ( $\lambda_{ex}/\lambda_{em}$ ) was inhibited by Cu, Zn superoxide dismutase (SOD) but not by catalase (data not shown). The rate constant for Mito-HE oxidation by  $O_2^{-}$  was calculated to be  $3.9 \pm 0.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$  and was independent of whether fluorescence was measured at excitation/emission wavelength pairs of 396/580 nm (selective for HO-Mito-Et<sup>+</sup>), 300/598 nm (selective for Mito-Et<sup>+</sup>) or 510/580 nm (which excites both products). This is consistent with the rate-limiting step in the competition for  $O_2^{-}$  being the initial oxidation of Mito-HE to a radical.

NMR spectra of purified O<sub>2</sub> oxidized Mito-HE (Fig. 4.11) indicated the O<sup>2</sup> product had lost two protons, H11, and either H2 or H9, which was consistent for the proposed structure of HO-Mito-Et<sup>+</sup>. Mass spectrometry of an  $O_2^{\bullet}$  oxidized sample of Mito-HE revealed ions at m/z = 316.0 and 630.5 (Fig. 4.5), which were identified as Mito-Et<sup>+</sup> from comparison with the mass spectrum of a Mito-Et<sup>+</sup> standard (Fig. 4.12). Mito-Et<sup>+</sup> can contaminate Mito-HE solutions and a small percentage of Mito-Et<sup>+</sup> can also be generated upon oxidation by xanthine/xanthine oxidase (Fig. 4.8). Mass spectrometry supported the structure of HO-Mito-Et<sup>+</sup> revealing a doubly charged species at m/z = 324.0 (Fig. 4.5) consistent with the calculated mass of HO-Mito-Et<sup>+</sup>, 647.79 amu. Because HO-Mito-Et<sup>+</sup> is doubly charged, it was surprising to observe a singly charged species at m/z = 646.4 (Fig. 4.5). This was resolved by attributing the m/z at 646.4 to the deprotonated form of HO-Mito-Et<sup>+</sup>,  $O=Mito-Et^+$  (Fig. 4.5). Collision induced fragmentation of the parent ion at m/z 646.4 supported the proposed structure of  $O=Mito-Et^+$  (Fig. 4.13).

To determine the extent to which this deprotonation may occur *in vivo*, we determined the  $pK_a$  of the HO-Mito-Et<sup>+</sup> hydroxyl group by measuring

fluorescence using 396/579 nm ( $\lambda_{ex}/\lambda_{em}$ ) over a pH range from 1 – 12. The data were fit using the Henderson-Hasselbalch equation, which revealed two p $K_a$ s at 2.1 and 10.4 (Fig. 4.6). A third p $K_a$  was determined to be 0.65 using fluorescence at  $\lambda_{ex}$  = 396 nm and  $\lambda_{em}$  = 795 nm, which is maximal at acidic pH (data not shown). The p $K_a$ s of 0.65 and 2.1 were assigned to the 8-ammonium and 3-ammonium groups of the phenanthridine ring, respectively, which are consistent with the previously determined p $K_a$ s of Et<sup>+</sup>, 0.8 and 2.0 (104). The p $K_a$  at 10.4 was consistent for an aromatic hydroxyl group. Moreover, the fluorescence emission of HO-Mito-Et<sup>+</sup> was maximal and independent of pH over the physiological range (Fig. 4.6).

#### 4.4 Discussion

Measurement of intracellular O<sup>2</sup> generation has been hampered by the lack of an assay that is sensitive enough to compete with endogenous SOD and yet selective for O2. Zhao et al. have shown that the specificity for detecting O<sub>2</sub> with HE can be greatly improved if the hydroxylated product is distinguished from Et<sup>+</sup> by HPLC (66). However, the overlap in the emission spectra makes separating the two products difficult by fluorescence microscopy (66, 67). In the present study, we have uncovered a simple means to verify the selectivity of  $O_2^{-1}$  detection with either HE or Mito-HE by comparing fluorescence at two different excitation wavelengths. Excitation at 396 nm allows more selective imaging of the hydroxylated products produced by O<sub>2</sub><sup>-</sup> and has greater sensitivity than the current practice of 510 nm excitation (Fig. 4.1). HO-Mito-Et<sup>+</sup> was detected using 396 nm excitation in both isolated mitochondria and cultured cells and fluorescence was increased with agents that increase O<sub>2</sub><sup>-</sup> formation, such as antimycin. However, the rate of fluorescence generation from antimycin-stimulated mitochondria was 31% greater at 510 nm versus 396 nm excitation, suggesting that Mito-Et<sup>+</sup> was being formed by non-superoxide dependent pathways and  $O_2^{-1}$  formation was overestimated using 510 nm excitation. Thus, monitoring fluorescence using

both excitation wavelengths can improve the selectivity for distinguishing  $O_2^{-1}$  from other cellular oxidative processes.

In 1934, Pauling and Neuman proposed the name superoxide based on the peculiar chemical bonding of KO<sub>2</sub> (105). However, superoxide more generally behaves as a mild reductant under physiological conditions rather than a "super"-oxidizing agent. The initial oxidation of the phenanthridine moiety by  $O_2^{-}$  is recognized to generate a radical intermediate (66, 67, 103, 106) and is among the more rapid reactions of  $O_2^{-1}$  with organic molecules: the rate constant for HE was estimated here to be 2 x 10<sup>6</sup> M<sup>-1</sup>.s<sup>-1</sup> and the oxidation of Mito-HE was twice as fast. This rate is about 500 times slower than the rate of O2<sup>-</sup> scavenging by SOD. However, the second reaction of the Mito-HE radical with O2<sup>-</sup> would involve radical-radical coupling to produce a hydroperoxide intermediate and should approach the diffusion limit (>10<sup>9</sup> M<sup>-1</sup>s<sup>-</sup> <sup>1</sup>). Hence, the Mito-HE radical could efficiently compete for O<sub>2</sub><sup>•</sup> with SOD in vivo to produce HO-Mito-HE. The hydroperoxide intermediate can spontaneously rearrange to lose a water molecule, yielding the hydroxylated product (Fig. 4.7). Hydroxylation appears to be relatively specific for O2 - as other common biological oxidants generated only a small percent of the fluorescent signal generated by  $O_2^{\bullet}$  (Table 4.1). Although the hydroxyl radical in theory could add to the radical to give a hydroxylated product, the hydroxyl radical is a promiscuous oxidant that is far more likely to react with the multitude of other organic molecules in a cell (107).

Most fluorescent probes used to image oxidants in cells, including reduced forms of fluorescein, rhodamine and ethidium, are susceptible to autooxidation through radical intermediates when illuminated (57). These probes can also be oxidized by a variety of intracellular peroxidases, oxidases or cytochromes to yield radical intermediates that dismutate to give fluorescent products (53-56). Therefore, the detection of the two-electron fluorescent product gives a rather nonselective assay of oxidative stress, yet is commonly used as a superoxide indicator. Paradoxically, oxidation of HE by these alternative oxidative mechanisms could enhance  $O_2^{-}$  detection by producing more HE radical, which increases the probability of trapping  $O_2^{-}$  to form HO-Et<sup>+</sup>.

The detection of HO-Et<sup>+</sup> is generally a semiquantitive assay for intracellular  $O_2^{\bullet}$  because the relative fraction of  $O_2^{\bullet}$  reacting with SOD will be unknown. A second confounding variable can be the endogenous production of nitric oxide competing for  $O_2^{\bullet}$ , which may need to be inhibited by nitric oxide synthase inhibitors for  $O_2^{\bullet}$  to be detectable. On the other hand, we have found that the addition of an exogenous nitric oxide donor can inhibit the oxidation of Mito-HE (not shown) and can be used as a control of selectivity for  $O_2^{\bullet}$ .

The accumulation of Mito-HE depends upon the mitochondrial membrane potential with an approximate ten-fold increase in uptake for every 60 mV increase in membrane potential (71). The mitochondrial membrane potential is typically in the range of -140 to -170 mV, which could concentrate Mito-HE in the mitochondria up to one thousand fold relative to the medium. Such high intramitochondrial concentrations should allow Mito-HE to compete with manganese SOD for  $O_2^{\bullet}$ . However, excessive Mito-HE accumulation may stress mitochondria and enhance their sensitivity to inhibitors or other mitochondrial targeted molecules. Potentiometric probes may be added at the end of the incubation with Mito-HE to assess general mitochondrial integrity.

Careful optimization of conditions is necessary when using ethidiumbased probes and several caveats must be considered. Mitochondrial depolarization may reduce the efficiency of  $O_2^{-}$  detection due to the decreased uptake of Mito-HE. Mito-HE itself, even at low (1 -10  $\mu$ M) concentrations, may disrupt membrane potential or increase mitochondrial permeability. We have observed a rapid loss of fluorescence from mitochondria and subsequent redistribution to the nucleus after incubation with as little as 2  $\mu$ M Mito-HE. The optimal concentration of Mito-HE should be determined empirically in each cell type, and varied in our experience from 0.1 - 0.5  $\mu$ M. Because DNA is necessary to enhance the fluorescence of HO-Mito-Et<sup>+</sup>, fluorescence might be limited by the amount of mitochondrial DNA, particularly in rho-naught cells or other pathological conditions where mitochondrial DNA has been deleted. Fluorescence intensity could artificially increase due to photooxidation. Photooxidation may be minimized by using the lowest possible concentration of Mito-HE and reducing the exposure to light throughout an experiment. Autofluorescence of cells will also be higher using 405 nm excitation and therefore should be controlled in microscopy experiments.

Because HE and Mito-HE accumulate to different extents within cells depending upon membrane potential, differences in fluorescence intensity between the two probes may not be directly compared to assess O2. production between the two compartments. Higher concentrations of HE may be needed to achieve cytosolic concentrations similar to those of Mito-HE in mitochondria. Also, it is possible that HE could be oxidized by mitochondrially generated O<sub>2</sub>. Selective inhibitors would be needed to uncover the source of O<sub>2</sub><sup>•</sup>. Exact quantitation of products using fluorescence microscopy is fraught with difficulties and therefore HPLC methods (67, 106) guantifying the relative amount of hydroxylated and non-hydroxylated products should be used in conjunction with fluorescence experiments when possible. If intracellular oxidation of Mito-HE to Mito-Et<sup>+</sup> should become more than 10-20 fold greater than HO-Mito-Et<sup>+</sup>, a majority of fluorescence from 396 nm excitation could be due Mito-Et<sup>+</sup>. However, monitoring the oxidation at both 396 and 510 nm will reveal this potential artifact.

In conclusion, with judicious choice of conditions and an appreciation for the limitations of these probes, we have shown that the oxidation of HE to HO-Et<sup>+</sup> and Mito-HE to HO-Mito-Et<sup>+</sup> can be a sensitive indicator to monitor dynamic changes of endogenous  $O_2^{-}$  generation.

## 4.5 Materials and Methods

MitoSOX Red<sup>™</sup> mitochondrial superoxide indicator (Mito-HE), Mito-Et<sup>+</sup> hydroethidine, and ethidium were obtained from Invitrogen-Molecular Probes, Inc. The purity of Mito-HE was ascertained by HPLC (as described below) because Mito-Et<sup>+</sup> can be a contaminant in Mito-HE. If Mito-Et<sup>+</sup> contamination was  $\geq$  10%, Mito-HE was either purified by HPLC or reduced by an equimolar amount of NaBH<sub>4</sub> (50 nmol in ethanol) with the reaction quenched by the addition of 120 nmol HCI. Recombinant human Cu, Zn superoxide dismutase was expressed in *Escherichia coli* (108). Xanthine oxidase activity was assayed by the reduction of cytochrome c ( $\Delta \varepsilon_{550}$ =21 mM<sup>-1</sup>cm<sup>-1</sup>) (109). Mito-HE was dissolved in DMSO to 5 mM and stored in the dark at -20°C for a maximum of three days. Mito-HE and all ethidium derivatives are carcinogenic and should be detoxified by reacting solutions diluted to  $\leq 0.5$  mg/ml ethidium with 0.2 volume 5% hypophosphorous acid and 0.12 volume of fresh 0.5 M sodium nitrite, mixing carefully and venting the nitrogen gas evolved. Dimethylsulfoxide (DMSO) penetrates gloves and skin and should also be used with caution.

Mito-HE and its oxidation products were separated using C<sub>18</sub> reverse phase HPLC (Supelco column, 15cm x 4.6 mm, 5  $\mu$ M) and a photodiode array detector. The mobile phase was H<sub>2</sub>O/CH<sub>3</sub>CN in 0.1% formic acid and 0.1 % trifluoroacetic acid, using a linear gradient from 30% to 35% organic over 40 min. Fluorescence spectra of 1.0  $\mu$ M HO-Mito-Et<sup>+</sup>, Mito-Et<sup>+</sup>, HO-Et<sup>+</sup>, and Et<sup>+</sup> were obtained using samples that were incubated for 15 minutes at 37°C with 1 mg/mL salmon sperm DNA, on a SLM Aminco 8100c Fluorometer. The extinction coefficients for HO-Mito-Et<sup>+</sup> and Mito-Et<sup>+</sup> were determined to be  $\epsilon_{478}$ =9,400 M<sup>-1</sup>cm<sup>-1</sup> and  $\epsilon_{488}$ =5,800 M<sup>-1</sup>cm<sup>-1</sup>, which were nearly identical to those reported for HO-Et<sup>+</sup> (110) and Et<sup>+</sup>.

A Spectra MAX Gemini fluorometer equipped with a 96 well plate reader was used for the SOD competition assays and also to compare fluorescence emission of Mito-HE oxidized by several oxidants. Assays were performed in 100 mM potassium phosphate buffer with 100  $\mu$ M DTPA, pH = 7.4, 37°C. To determine rate constants, fluorescence emission was plotted as a function of the fractional inhibition (f<sub>i</sub>) by the addition of SOD, as described by Eq 1.

$$f_i = k_{Et}[HE] / (k_{Et}[HE] + k_s[SOD])$$
(1)

The rate of  $O_2^{-1}$  scavenging by SOD (k<sub>s</sub>) has been determined as  $1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  (111).

Superoxide was generated by xanthine oxidase where the added volume of xanthine oxidase suspension was adjusted to generate 0.2 nM  $O_2^{-1}$ /sec with 0.5 mM xanthine, as measured by cytochrome *c* reduction. Alternatively, 10  $\mu$ M Mito-HE was reacted with five 100  $\mu$ M additions of peroxynitrite ± 25 mM ammonium bicarbonate with immediate vortexing, 100  $\mu$ M hydrogen peroxide or 100  $\mu$ M hypochlorous acid. Hydroxyl radical was generated using xanthine oxidase, 0.5 mM xanthine, and 10  $\mu$ M Fe<sup>3+</sup>-EDTA. All assays were repeated at least three times and are reported ± the standard deviation.

The uptake of Mito-HE into isolated mitochondria was determined using an electrode selective for the triphenylphosphonium cation (112, 113). Rat livers were homogenized in 250 mM sucrose, 5 mM Tris-HCl (pH 7.4), and 1 mM EGTA, followed by differential centrifugation (114). The electrode was constructed as described previously (112), and the voltage was measured relative to an Ag/AgCl reference electrode. The electrode was calibrated by the sequential addition of 5 x 1  $\mu$ M reduced Mito-HE to a stirred thermostatted chamber at 30°C, containing 3 mL KCl buffer (120 mM KCl, 10 mM HEPES at pH 7.2), 1 mM EGTA) and 4 µg/mL rotenone ± rat liver mitochondria (0.5 mg protein/mL). Mitochondria were energized with succinate (10 mM), and the membrane potential was subsequently dissipated with 0.5 μM carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP). Association of Mito-HE with mitochondria was measured as a decrease in the Mito-HE concentration in the medium.

To test the fluorescence emission of Mito-HE in isolated mitochondria, rat heart mitochondria were isolated as described before (115, 116). Mitochondria were tested for coupled respiration with an oxygen electrode and Mito-HE oxidation by mitochondria was followed with the SLM Aminco 8100c Fluorometer ( $\lambda_{exc}$ =396, 510nm;  $\lambda_{em}$ =579 nm) for 10 min. Mitochondria (600 µg protein/mL) were incubated with 10 mM succinate, 8.25 µM rotenone, 2.5 mM ADP and 0.8µM Mito-HE with stirring at 30°C. The respiration buffer was 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris HCl, 5 mM KH<sub>2</sub>PO<sub>4</sub>, and 0.1% fatty acid free bovine serum albumin, pH = 7.2. Antimycin (15 µM) was added after 1 min to stimulate O<sub>2</sub><sup>--</sup> generation. Rates of fluorescence increase in the presence of mitochondria were converted to approximate rates of O<sub>2</sub><sup>--</sup> generation by comparison with a 0.8 µM Mito-HE standard oxidized by a known rate of superoxide (7.2 nM O<sub>2</sub><sup>--</sup>/sec generated with 130 µM xanthine and 2.5 µL xanthine oxidase) in the presence of 50 µg salmon sperm DNA.

Primary oligodendrocyte cultures were prepared from 1 day old Sprague-Dawley rat pups as previously reported (117). Cultured cells were transferred to the heated stage (37°C) of a Zeiss LSM510 confocal microscope with constant 5% CO<sub>2</sub>. Live cells were imaged with a 63X oil immersion objective using either 405 nm or 514 nm laser excitation. A single field of view was used throughout an entire experiment and corrected for autofluorescence by collecting 405 nm excitable signal before labeling with Mito-HE. Image acquisition conditions were kept constant for comparison between ± antimycin. Cells were incubated with 0.1  $\mu$ M Mito-HE for 15 minutes, washed and incubated in supplemented L15 media (117). Images were obtained immediately after Mito-HE addition and every 10 min thereafter. In some experiments, antimycin (15  $\mu$ M) was added immediately after Mito-HE

with 3.6 nM MitoTracker<sup>™</sup> Deep Red (Invitrogen Corp.) to visualize mitochondrial colocalization and general integrity.

To determine the chemical structure of the oxidation product, samples were directly injected onto the electrospray interface of an LC-Q Classic ion trap mass spectrometer in positive ion mode. The mobile phase was 25% 10mM ammonium acetate and 75% acetonitrile with  $5\mu$ L / min flow rate. The source voltage was 2.72 kV and the capillary voltage was 32.7 V.

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## 4.6 Figure Legends

Figure 4.1 HO-Mito-Etd<sup>+</sup> has a selective excitation wavelength at 396 nm. (a) The fluorescence emission of an equimolar concentration of Mito-Et<sup>+</sup> (—) overlapped 40% of the fluorescence emission of HO-Mito-Et<sup>+</sup> (---) exciting at 510 nm. (b) The excitation spectrum (emission at 579 nm) revealed HO-Mito-Et<sup>+</sup> (---) has a distinct excitation at 396 nm that is not present for Mito-Et<sup>+</sup> (—). (c) Excitation at 396 nm enhanced the fluorescence emission of HO-Mito-Et<sup>+</sup> (—) by 70% and reduced spectral overlap from Mito-Et<sup>+</sup> (—) to 10%, as compared with 510 nm excitation.

Figure 4.2 Mito-HE was taken up by isolated mitochondria in a manner dependent upon mitochondrial membrane potential, as well as by adsorption to membranes. An ion-selective electrode was used to measure the concentration of Mito-HE in a mitochondrial suspension. Mito-HE (five sequential additions of 1  $\mu$ M; arrowheads) was added to energized mitochondria and the mitochondrial uptake of Mito-HE was measured as a decrease in free Mito-HE. The uncoupler FCCP (0.5  $\mu$ M) was added to induce mitochondrial uncoupling and Mito-HE release (measured as an increase in free Mito-HE). In (A), Mito-HE was added to the chamber before the mitochondria, enabling an estimation of the extent of non-potential dependent

mitochondrial association. In (B), mitochondria were present in the chamber before Mito-HE addition.

Figure 4.3 The fluorescence emission of isolated mitochondria incubated with Mito-HE. Each trace was evenly plotted above the previous for clarity and the rate of Mito-HE oxidation was calculated from the approximately linear slope of the first 60 seconds. (a) 0.8  $\mu$ M Mito-HE incubated without mitochondria did not show an increase in fluorescence at  $\lambda_{ex}$ =396 nm, even upon the addition of 15  $\mu$ M antimycin (arrow). (b) Fluorescence using  $\lambda_{ex}$ =396 nm of mitochondria respiring in State 3 with 0.8  $\mu$ M Mito-HE. (c) Fluorescence using  $\lambda_{ex}$ =510 nm of mitochondria respiring in State III with 0.8  $\mu$ M Mito-HE. (d) The same as (b) with  $\lambda_{ex}$  = 396 nm with the addition of 15  $\mu$ M antimycin (arrow). (e) The same as (d) except  $\lambda_{ex}$ =510 nm. (f) 0.8  $\mu$ M Mito-HE without mitochondria, was oxidized by xanthine/xanthine oxidase (generating 7.2 nM O<sub>2</sub><sup>-</sup>/sec) in the presence of 50  $\mu$ g DNA. The plotted curves represent 3 – 10 replicates of each experiment. Ethanol alone had no effect.

Figure 4.4 Fluorescence from cultured oligodendrocytes incubated with Mito-HE using the two different excitation wavelengths, 405 nm (blue) and 514 nm (red). Instrument parameters were set and held constant to minimize autofluorescence at 405 nm, which is generally greater than at 514 nm. (a) Oligodendrocyte after a 15 minute incubation with 0.1  $\mu$ M Mito-HE using  $\lambda_{ex}$ =405 nm and then 40 minutes later (b). The same oligodendrocyte using  $\lambda_{ex}$ = 514 nm (c and d). Fluorescence from oligodendrocytes was enhanced with 15  $\mu$ M antimycin at  $\lambda_{ex}$ =405 nm (e) and 40 minutes later (f). The antimycin-stimulated increase in fluorescence was also observed using  $\lambda_{ex}$ =514 nm (g and h).

Figure 4.5 Mito-HE oxidized by xanthine/xanthine oxidase was analyzed by electrospray ionization and ion trap mass spectrometry. The ions at m/z = 316.0 and 630.5 were identified as Mito-Et<sup>+</sup>. The doubly charged ion at m/z = 324.0 corresponded to the molecular weight of the hypothesized structure of HO-Mito-Et<sup>+</sup>. The singly charged ion at m/z = 646.0 lead to the proposal of the carbonyl structure, O=Mito-Et<sup>+</sup>.

Figure 4.6 The fluorescence emission of HO-Mito-Et<sup>+</sup> in the presence of 0.17 µg DNA as a function of pH ( $\lambda_{ex}$  = 396 nm;  $\lambda_{em}$  = 579 nm). This curve was fit to find two pK<sub>a</sub>s at 2.1 and 10.5 and a third pK<sub>a</sub> was determined to be 0.65 by plotting the fluorescence emission at 795 nm with  $\lambda_{ex}$ =396 nm (not shown). Fluorescence of HO-Mito-Et<sup>+</sup> is maximal and independent of pH over the physiological range.

Figure 4.7 Proposed scheme for the oxidation of Mito-HE (R =  $(CH_2)_6P^+(Ph)_3$ ) or HE (R =  $CH_2CH_3$ ) by  $O_2^-$ . The radical intermediate is shown as a cation (HE<sup>+</sup>), but it could alternatively be a neutral radical (Et<sup>-</sup>).

## Supplemental Figures & Table Legends.

Figure 4.8 Reverse-phase HPLC separation of Mito-HE (a) indicated that Mito-HE (13.2 min) is contaminated with Mito-Et<sup>+</sup> (29.2 min) and also HO-Mito-Et<sup>+</sup> (30.2 min). When Mito-Et<sup>+</sup> is reduced to Mito-HE, a small amount of superoxide may be generated by the NaBH<sub>4</sub> reduction of oxygen causing the small contamination of HO-Mito-Et<sup>+</sup>. Xanthine/xanthine oxidase oxidized Mito-HE (b) increased the superoxide product, HO-Mito-Et<sup>+</sup> with little increase in Mito-Et<sup>+</sup>. HPLC traces represent absorbance at 254 nm and the area of the peak is printed next to the peak.

Figure 4.9 (a) The fluorescence emission of  $Et^+(-)$  overlaps 45% of the fluorescence emission from HO- $Et^+$  (---) while exciting at 510 nm. (b) The excitation spectra (emission at 579 nm) revealed HO- $Et^+$  (---) has a distinct absorption at 396 nm that is not present for  $Et^+$  (--). (c) Excitation at 396 nm enhanced the fluorescence emission of HO- $Et^+$  (---) by 55% and attenuated the emission overlap from  $Et^+$  (--) to only 10%.

Figure 4.10 Live bovine pulmonary artery endothelial cells were incubated with 0.5  $\mu$ M Mito-HE in warm Hanks balanced salt solution (HBSS) for 10 min at 37° C before washing twice in warm HBSS and imaging. Imaging was performed on a Zeiss Axiovert 200M inverted fluorescence microscope equipped with a mercury arc lamp for widefield illumination, a CoolSNAP HQ CCD camera, and MetaMorph for hardware control and image acquisition. The HO-Mito-Et<sup>+</sup> product was excited using 387+/- 5.5 nm or 484 +/- 5.0 nm excitation filters mounted in a filter wheel. The 510 nm dichroic mirror and 580 nm +/- 30 nm emission filter were mounted in a cube within the microscope. Samples were maintained at 37° C on the stage and images were acquired using a Zeiss C-Apochromat 63X 1.2 N. A. water immersion objective. Images shown were acquired 45 min after addition of probe and represent signal detected when exciting at 387 nm (green) or 484 nm (red) for 8 sec at each wavelength.

Figure 4.11 The 2-D COSY NMR spectrum of HO-Mito-Et<sup>+</sup> (b) compared to Mito-HE (a) indicated a loss of two protons, H11 and also H2 or H9. It was not possible to determine between the two sides of the phenanthridine ring. Therefore, one side of the ring was ambiguously assigned, showing the loss of H9 in (b) because this is the more reactive side of ethidium in substitution reactions and H9 would be less hindered sterically. A Bruker DPX, 400 MHz NMR was used to obtain spectra of samples purified by HPLC and reconstituted in deuterated DMSO. HSQC spectra using the natural abundance of <sup>13</sup>C were also obtained to aide in proton assignments (data not shown).

A large scale synthesis of HO-Mito-Et<sup>+</sup> was accomplished using Fremy's salt (nitrosodisulfonate radical dianion). Briefly, the inside of a 1.5 mL eppendorf tube was coated with ~2 mg of fremy's salt. A 1mL solution of 500  $\mu$ M Mito-HE diluted in DMSO and 10% acetic acid (1:1 ratio) was then added to the eppendorf tube and rapidly vortexed. This was repeated in five different aliquots and the resulting solutions were combined and purified by a Varian large scale HPLC system equipped with a 250 X 21.4 mm (L X ID) C<sub>18</sub> column. The mobile phase was H<sub>2</sub>O/CH<sub>3</sub>CN in 0.1% formic acid and 0.1 % trifluoroacetic acid, using a linear gradient from 30% to 50% organic over 15 min at 8mL/min. Purified fractions were collected and dried *in vacuo*.

Figure 4.12 A standard solution of Mito- $\text{Et}^+$  produced ions at m/z = 315.9 and 630.3 which can be explained by the deprotonation of the doubly charged species to a singly charged species.

Figure 4.13 The ion at m/z of 646.0 from  $O_2^{-}$  oxidized Mito-HE was fragmented by a second quadrupole to give the MS<sup>2</sup> spectrum in (a) which supported the proposed structure of O=Mito-Et<sup>+</sup>. Interpretation of the spectrum is shown in (b).

Table 4.1 Selectivity of oxidation of Mito-HE is expressed relative to the fluorescence generated by  $O_2^{-}$ . Oxidant exposure ( $\mu$ M•min) represents the area under the curve (AUC) or integrated amount of oxidant exposure over time to account for differences in half life decay of oxidants. A significant concentration of DMSO (28 mM) from the Mito-HE stock, as well as urate, xanthine or xanthine oxidase were all present in the hydroxyl radical generating system and will therefore have competed with the reaction of Mito-HE for hydroxyl radical. The increase in fluorescence of 10  $\mu$ M Mito-HE upon oxidant exposure was measured using  $\lambda_{ex}$ =396 and  $\lambda_{ex}$ = 510 nm with  $\lambda_{em}$  = 580 nm. Mito-HE appears to be selectively oxidized by superoxide. For example, a 28-fold greater exposure to hydrogen peroxide was required to obtain just 10% of the superoxide-induced fluorescent signal.

4.7 Figures

Figure 4.1



Figure 4.2



Figure 4.3.





Figure 4.5


Figure 4.6



Figure 4.7





Figure 4.8

Figure 4.9



Figure 4.10











4







+H⁺

Calculated m/z = 630.78 amu

















Table 4.1

| Oxidant   | Oxidant<br>AUC | Mito-HE<br>λex <b>=396nm</b>      | HE<br>λex <b>=396nm</b>           | Mito-HE<br>λex <b>=510nm</b>      | ΗΕ<br>λex <b>=510nm</b>         |
|---|----------------|-----------------------------------|-----------------------------------|-----------------------------------|---------------------------------|
| Superoxide<br>(O₂ <sup>⊷</sup> )                                  | 3.52           | $100\pm5.5\%$                     | $100\pm35\%$                      | $100\pm5.3\%$                     | 100 ± 21%                       |
| Peroxynitrite<br>(ONOO <sup>-</sup> )                             | 8.33           | $2.2\pm0.3\%$                     | $1.5\pm0.5\%$                     | $\textbf{3.0} \pm \textbf{0.9\%}$ | $0.6\pm0.2\%$                   |
| Nitrosoperoxy<br>carbonate<br>(ONOOCO <sub>2</sub> <sup>-</sup> ) | 8.33           | 2.1 ± 0.4%                        | $1.6\pm0.6\%$                     | $1.6\pm0.4\%$                     | $1.2\pm0.3\%$                   |
| Hydrogen<br>Peroxide<br>(H <sub>2</sub> O <sub>2</sub> )          | 100            | $\textbf{6.4} \pm \textbf{0.5\%}$ | $\textbf{6.2} \pm \textbf{2.2\%}$ | $10\pm3.8\%$                      | 6.7 ± 1.4%                      |
| Hydroxyl<br>Radical<br>(•OH)                                      | -              | $\textbf{4.4} \pm \textbf{0.2\%}$ | $1.3\pm0.5\%$                     | $\textbf{6.0} \pm \textbf{1.2\%}$ | $\textbf{2.2}\pm\textbf{3.2\%}$ |
| Hypochlorous<br>Bleach<br>(HOCl)                                  | 100            | $\textbf{4.8} \pm \textbf{0.2\%}$ | $\textbf{3.3} \pm \textbf{1.2\%}$ | $\textbf{8.4}\pm\textbf{0.1\%}$   | $4.3\pm0.9\%$                   |

Chapter 5

The detection of mitochondrially-generated superoxide.

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Invited chapter for submission to Current Protocols in Toxicology

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#### 5.1 Introduction

The reduction of molecular  $O_2$  to the small free radical, superoxide, has been implicated in a number of pathological disorders including neurodegenerative diseases (6, 36-40), aging (36-38, 43, 44), cancer (37), and diabetes (41). In toxicology, superoxide is associated with the toxic effects observed with the metabolism of xenobiotics (118). The formation of superoxide and the quinone radical are associated with the cardiotoxicity of the guinone anticancer agents, daunomycin and adriamycin (119). Pulmonary edemia toxicity from antibacterial agents such as nitrofurantoin, nitrofurzone and metromidazole are associated with superoxide generation (120). In addition, superoxide production is associated with the neurotoxin, 6hydroxydopamine, the hemolytic anemia associated with phenylhydrazine and the pulmonary toxicity of paraquat (118). A number of redox active, organic compounds, such as paraguat, nitrofurantoin, adriamycin and daunomycin, interact with superoxide to release iron from the NADPH-cytochrome P450 reductase, facilitating the generation of the damaging oxidant, the hydroxyl radical (118).

Superoxide can be generated enzymatically under certain conditions, for example by NADPH oxidase, xanthine oxidase, and nitric oxide synthase. However, superoxide generation is largely associated with the mitochondria. Mitochondria consume ~90% of respired oxygen where 0.1 to 8% of the electrons shuffled through their respiratory chain leak to generate superoxide. Therefore mitochondria have become a leading culprit in superoxide generation and oxidative stress.

Thus, a selective method to detect mitochondrially generated superoxide would be extremely valuable. The current methods available to detect superoxide are limited by technical difficulties and are greatly disposed with the potential to give false positives. Some chemiluminescent  $O_2^{-}$  detectors actually generate  $O_2^{-}$  by either uncoupling the respiratory chain or reacting with oxygen (e.g. luminol) (36, 41). Fluorescent probes are another

widely used tool for superoxide detection. However fluorescent detectors, including reduced forms of fluorescein, rhodamine and ethidium, are susceptible to autooxidation through radical intermediates when illuminated (57) and can also be oxidized by a variety of intracellular peroxidases, oxidases or cytochromes to yield radical intermediates that dismutate to give fluorescent products (53, 54, 56, 65). Therefore, the detection of their two-electron fluorescent products gives a rather nonselective assay of oxidative stress, yet is commonly used as a superoxide indicator.

Hydroethidine (HE) is one of the most common fluorescent probes to detect superoxide although its chemistry was misunderstood for a number of years. Contrary to the popular notion that superoxide would oxidize HE by two electrons to ethidium, superoxide can actually oxidize HE to a hydroxylated product (HO-Et<sup>+</sup>; Scheme 1) (66).



Therefore, selective detection of the hydroxylated product versus ethidium would provide a more selective assay for the detection of superoxide generated in the cytosol (66, 67).

To detect mitochondrial superoxide, HE was covalently joined through a hexyl linker to a triphenyl phosphonium group (Mito-HE). Mito-HE is commercially available as MitoSOX Red<sup>TM</sup> (Invitrogen Corp). Mito-HE behaves chemically similar to HE and is also selectively oxidized by superoxide to a hydroxylated product (HO-Mito-Et<sup>+</sup>). It is important to be able to distinguish the hydroxylated products from other products because both HE and Mito-HE are also susceptible to intracellular, non-specific oxidation which can generate their fluorescent, two electron oxidized products, ethidium (Et<sup>+</sup>), and Mito-ethidium (Mito-Et<sup>+</sup>). Methods currently in use for superoxide

detection using HE suggest 500 nm excitation, which induces fluorescent emission from both the ethidium products and the hydroxylated products due to their spectral overlap. Therefore 500 nm excitation does not distinguish between oxidation of HE by superoxide from other non–selective intracellular oxidation of HE. We discovered the superoxide product, HO-Mito-Et<sup>+</sup> has a distinct excitation wavelength at 396 nm that is not present for Mito-Et<sup>+</sup> (121). Excitation at 396 nm enhances fluorescence emission of HO-Mito-Et<sup>+</sup> by 70% and decreases the emission overlap with Mito-Et<sup>+</sup> to 10% (121).

With careful attention to experimental details and with optimization of experimental conditions, the detection of HO-Mito-Et<sup>+</sup> using 396 and 500 nm exciation can be a powerful tool to detect mitochondrially generated superoxide (121). The same excitation wavelengths can be used with HE to detect cytosolic superoxide, as hydroxy-ethidium also possesses the distinct excitation at 396 nm (121). This paper will focus on methods using the mitochondrially-targeted derivative, Mito-HE, and detecting mitochondrially generated superoxide. HE can be selectively used to detect superoxide through separation and quantification of HE oxidation products by HPLC (66, 67). Oxidized HE will accumulate into the nucleus and therefore live cell imaging cannot reveal the site of superoxide generation. Unfortunately, analysis of HE oxidation by either HPLC or fluorescence microscopy will not reveal the site and source of superoxide generation. Mito-HE is advantageous as a consequence of its direct targeting to the mitochondria in which compartment it enables the assessment of superoxide production.

#### 5.2 Protocols

# FLUORESCENT DETECTION OF SUPEROXIDE USING A SELECTIVE EXCITATION WAVELENGTH.

The detection of mitochondrially-generated superoxide using the fluorescent probe, Mito-HE, is possible in several systems including isolated mitochondria

and cultured cells. The two excitation wavelengths can be used in conjunction to selectively detect the hydroxylated product (using 396 nm excitation) and also total intracellular oxidation (using 510 nm excitation).

## BASIC PROTOCOL 1. The detection of superoxide from isolated mitochondria.

This method allows for the selective detection of superoxide from isolated mitochondria. Experimental mitochondria can be isolated from different *in vivo* models, or mitochondria from one experimental system can be tested with reagents which might stimulate superoxide generation.

# Materials

Isolated mitochondria

Mitochondrial Respiration Buffer (see recipe in *Reagents and Solutions*)

MitoSOX Red<sup>™</sup> (Mito-HE)

50 mM Potassium Phosphate buffer, pH = 7.4

0.8 mM antimycin in ethanol

0.6 M Succinate

1.65 mM Rotenone in DMSO

85 mM ADP

- Turn on the fluorometer and allow it to stabilize ~30 min prior to use. Set wavelengths to 396 nm excitation and 579 nm emission in kinetics mode and set the thermostatting device (i.e., cuvette temperature) to 30°C. Adjust high voltage so the fluorescent signal is within a detectable range.
- 2. Pre-warm buffer to 30°C.
- 3. Determine the concentration of mitochondrial protein in isolated mitochondrial suspensions using the BioRad DC protein assay kit, per manufacturer's instruction.
- 4. Prepare Mito-HE solution by dissolving 50  $\mu$ g of Mito-HE in 20  $\mu$ L of DMSO for a 3.96 mM solution. Dilute 2.02  $\mu$ L of the 3.96

mM solution into 100  $\mu$ L (final) of potassium phosphate buffer for an 80  $\mu$ M solution of Mito HE.

- 5. Place 16.7  $\mu$ L of 0.6 M succinate in a 1 mL cuvette with stir bar.
- 6. Add 29.4 µL of 85 mM ADP
- 7. Add 5 µL of 1.65 mM Rotenone
- 8. Add 10  $\mu$ L of 80  $\mu$ M Mito-HE
- Calculate the volume of mitochondrial suspension required to have a final concentration of 600 µg/mL mitochondrial protein in 1 mL. Then add an appropriate volume of respiration buffer to the cuvette so that the final volume in the cuvette (after mitochondrial suspension is added) will be 1 mL.
- 10. Place cuvette in the fluorometer and turn on the magnet to stir the solution.
- 11. Begin recording emission every second to obtain a baseline reading for ~40 sec. *This trace should be level.*
- 12. Close shutters and quickly open the fluorometer and add the mitochondrial suspension, checking that the stir bar is still stirring. Then quickly close the fluorometer and open shutters. Do not stop recording fluorescence emission during this time in order to record any initial bursts in superoxide immediately after the addition of mitochondria.
- 13. Incubate ~ 50 seconds.
- If desired, add 18.8 μL of 0.8 mM antimycin, or other superoxide stimulator of interest.
- 15. Record fluorescence emission for an additional time period (1 10 min).

After this time the mitochondria are likely to become anaerobic (see Critical Parameters).

 Convert rates of fluorescence emission to nmoles superoxide/ min (See Supplemental Protocol 1). 17. Repeat using 510 nm excitation.

## Supplemental Protocol 1.

# Converting rates of fluorescence emission to nanomoles superoxide per minute using a xanthine/xanthine oxidase standard.

The quantity of superoxide generated by xanthine/xanthine oxidase can be measured using the reduction of cytochrome *c* ( $\Delta \epsilon_{550}$ =21 mM<sup>-1</sup>cm<sup>-1</sup>) (109).

### Materials

Mito-HE

Xanthine oxidase suspension (see recipe in *Reagents and Solutions*)

1.3 mM Xanthine

10 mg/mL Salmon sperm DNA

# Fluorometer

- Turn on fluorometer and allow to stabilize ~30 min prior to use. Set wavelength to 396 nm excitation and 579 nm emission in kinetics mode and thermostatting device (i.e., cuvette temperature) to 30°C. Adjust high voltage so the fluorescent signal is within a detectable range.
- Prepare Mito-HE solution by dissolving 50 μg of Mito-HE in 20 μL of DMSO for a 3.96 mM solution. Dilute 2.02 μL of the 3.96 mM solution into 100 μL (final) of potassium phosphate buffer for an 80 μM solution of Mito HE.
- 3. Add 10  $\mu$ L of 80  $\mu$ M Mito-HE to a 1 mL cuvette with stir bar.
- 4. Add 50 µL of 1.3 mM xanthine
- 5. Add 5 µL of 10 mg/mL Salmon sperm DNA
- 6. Add an appropriate volume of respiration buffer for a final volume of 1 mL.
- 7. Place cuvette in fluorometer and turn on the magnet to stir the solution.
- Begin recording emission every second to obtain a baseline reading for ~40 sec. This trace should be level.

Close shutters and quickly open the fluorometer and add the xanthine oxidase suspension (generating 7.2 nM O<sub>2</sub><sup>\*</sup>/sec; or other rate determined from cytochrome *c* assay) checking that the stir bar is still stirring. Then quickly close fluorometer and open shutters.

Do not stop recording fluorescence emission during this time in order to record any initial bursts in superoxide.

- Record fluorescence emission for an additional time period (1 10 min).
- 11. Calculate rate of fluorescence emission. Use for experimental conversions.

#### BASIC PROTOCOL 2. The detection of superoxide in live cells using confocal microscopy.

Detection of intracellular superoxide utilizing Mito-HE is best performed using live cells with actively respiring mitochondria. Collecting fluorescent emission from two different excitation wavelengths provides a more selective method of superoxide detection than the conventional use of 500 nm excitation only. Following live cell imaging, cells can either be stained directly for mitochondria and nuclei (Support Protocol 3) or immunohistochemistry can be performed directly on the microscope to obtain additional information about the cells under investigation (Support Protocol 4).

#### Materials

Cultured cells of interest Mito-HE Warmed media Dulbecco's phosphate buffered saline (PBS) Confocal microscope equipped with: Diode (UV) laser with 405 nm excitaiton

Argon laser with 514 nm excitation

- 1. Turn on confocal microscope and lasers.
- Set up for live cell imaging using a heated stage with incubation chamber (37°C), a heater upon a 40X oil immersion objective and a constant flux of 5% CO<sub>2</sub>.
- 3. The confocal microscope should be set to collect fluorescence emission from two different excitation wavelengths. 405 nm excitation can be accomplished using a diode laser (set to 10% transmission) passing through a main dichroic and secondary beam splitter and emission collected using high pass filter, passing wavelengths higher than 560 nm to the detector. 514 nm excitation can be accomplished using an Argon laser (set to 6.1% transmission) passing through a main dichroic and secondary beam splitter, again collecting emission with a high pass filter, passing wavelengths higher than 560 nm to the detector.
- 4. Transfer live cells to the heated stage of the microscope.
- 5. Choose one field for the experiment, or choose several fields and mark the coordinates of that field in order to return to the same field later.
- 6. Obtain autofluorescence images of chosen field(s) using 405 and 514 nm excitation. Adjust the detector gain and amplifier offset values appropriately in order to minimize any emitting autofluorescence. It is advantageous to avoid fields of extremely high autofluorescence. The detector gain and offset values will have to be decreased in highly autofluorescent fields and this will cause a loss in sensitivity for Mito-HE fluorescent changes.
- Prepare Mito-HE solution by dissolving 50 μg of Mito-HE in 20 μL of DMSO for a 3.96 mM solution. Dilute 1 μL of the 3.96 mM solution into 10 μL PBS (final volume) for a 396 μM solution. Add 2.27 μL of the 396 μM solution to the cells on the

microscope (suspended in 3 mLs of media) for a final concentration of 0.3 µM Mito-HE. *The optimal concentration of Mito-HE will vary with cell type and should be determined empirically; see Critical Parameters and Troubleshooting.* 

8. Incubate cells with Mito-HE for 20 min at  $37^{\circ}C$  (5% CO<sub>2</sub>).

- 9. Aspirate media and replace with 2 mLs of new media.
- 10.Return to fields where autofluorescence images were already obtained and image fluorescence emission from both 396 and 514 nm excitation.
- 11. Incubate cells an additional 40 min at 37°C (5% CO<sub>2</sub>).
- 12. Return to fields of interest and image fluorescence emission from both 396 and 514 nm excitation to assay for a change in fluorescence emission.
- After imaging Mito-HE, it is possible to continue with live cell imaging of the mitochondria and nuclei (Support protocol 2) or with immunohistochemistry performed directly on the microscope (Support protocol 3).

# Support Protocol 2 Identification of mitochondria and nuclei

In order to confirm fluorescence emission from oxidized Mito-HE originated from the mitochondria, these organelles can be stained using MitoTracker Deep Red<sup>TM</sup>. MitoTracker Deep Red fluorescence emission will not interfere with the signal from Mito-HE. However, high concentrations of Mito-HE could dissipate the mitochondrial membrane potential, inhibiting the accumulation of MitoTracker Deep Red. Therefore low, optimal concentrations of Mito-HE should be used (see Troubleshooting).

# Materials.

MitoTracker<sup>™</sup> Deep Red DAPI (4,6 diamidino-2-phenylindole dihydrochloride) Fluorescent microscope equipped with excitation at far red (633 nm)

- 1. Dissolve 50  $\mu$ g into 500  $\mu$ L of DMSO.
- 2. Dissolve 10 mg DAPI into 1 mL DMSO.
- Add 0.2 µL of Mitotracker Deep Red directly to the cells on the stage of the confocal microscope after they have been analyzed with Mito-HE (final concentration of MitoTracker<sup>™</sup> Deep Red in media should be ~3.6 nM).
- 4. Add 0.8 μL of DAPI directly to the same cells: mix by gentle pipetting.
- 5. Incubate for 5 min.
- Image DAPI using 405 nm excitation from a diode laser (10% transmission) and collect emission using a band pass filter passing wavelengths from 420 480 nm to the detector.
- Image MitoTracker<sup>™</sup> Deep Red using 633 nm excitation (30% transmission) and collect emission using a high pass filter passing wavelengths higher than 650 nm to the detector.
- 8. Return to and image each field already assayed for superoxide generation using Mito-HE.

## Support Protocol 3 Immunohistochemistry following Live Cell Imaging

Immunohistochemistry can be performed directly on the stage of a confocal microscope. Because superoxide imaging with Mito-HE should be conducted on live cells, other information can be learned from immunohistochemistry by fixing cells directly on the microscope then being able to view the same fields already marked and imaged for Mito-HE. For example, antibodies could reveal cellular identification with marker proteins indicating which type of cells are generating superoxide in co-cultures. Identification of proteins possibly involved in superoxide generation could be addressed using immunohistochemistry and colocalization of protein with the superoxide signal can be ascertained. It is crucial when using this method to not move the dish

on the confocal stage. This allows the investigator to image same fields previously imaged with Mito-HE.

### Materials

Primary and Secondary Antibodies of Interest Blocking buffer 20% formaldehyde (refrigerated and protected from light) PBS

0.3% Triton X100 in PBS

DAPI

- Directly before use, dilute 0.4 mLs of 20% formaldehyde into 2 mLs (final volume) of PBS making at 4% solution of formaldehyde. *Diluted formaldehyde solutions will decay, use fresh.*
- After cells have been imaged with Mito-HE, aspirate off the media (leaving cell containing vessel on the stage of the microscope).
- 3. Add the 2mLs of 4% formaldehyde to the cells.
- Incubate for 15 min keeping cells under live cell imaging conditions (37°C) in a covered dish. It is important to keep the dish covered as small volume solutions will evaporate during longer incubations.
- Aspirate off fixative and wash gently by slowly pipetting 2 mLs of PBS into dish. Incubate each PBS wash for 5 min.
- 6. Repeat 5 min PBS wash for a total of three washes.
- 7. Permeabilize by adding 2 mLs of 0.3% TritonX 100 solution.
- 8. Aspirate and add the blocking buffer.
- 9. Incubate 50 min.

- 10.Aspirate and add the primary antibody diluted in 0.3% TritonX 100 solution. *Primary antibody dilutions and incubation times should be determined empirically.*
- 11. Aspirate and wash twice with PBS for 5 min each.
- 12.Dilute Secondary antibody appropriately in blocking buffer. Secondary antibody dilutions should be determined empirically.
- 13.Add Secondary antibody and incubate. Secondary antibody incubation times should be determined empirically.
- 14. Aspirate and wash twice with PBS for 5 min each.
- 15. Resuspend cells in 2mLs of PBS.
- 16.Add 0.8 µL DAPI and incubate 5 min.
- 17.Return to fields marked and previously imaged for oxidized Mito-HE, and image for antibody using excitation/emission wavelengths appropriate for the secondary antibody.
- 18.Image DAPI using 405 nm excitation from a diode laser (10% transmission) and collect emission using a band pass filter passing wavelengths from 420 480 nm to the detector.
- 19.Return to and image each field previously assayed for superoxide generation, readjusting the focal plane. *It is more than likely that the dish will have moved during this process, and the focusing may need to be readjusted to find the optimal focal plane for imaging.*

#### Support Protocol 4 DETANONOate control

Excitation at 396 nm is performed to increase selectivity for the superoxide product. However additional selectivity can be accomplished by inhibiting fluorescence with a superoxide scavenger. DETANONOate releases nitric oxide in a steady state manner and nitric oxide readily diffuses through cellular membranes and reacts with superoxide at rates limited only by

diffusion (~ $10^9$ M<sup>-1</sup>s<sup>-1</sup>). Other superoxide scavengers, such as FeTCCP, Tiron and MnTBAP, may actually inhibit fluorescent signals not by scavenging superoxide, but by reacting directly with and reducing the hydroethidine radical. DETANOnoate is also advantageous to MnTBAP because MnTBAP can confound experiments by reacting directly with HE to generate Et<sup>+</sup>, and also by absorbing 470 nm excitation used to detect fluorescence from the hydroxy-product (122).

## Materials

3.68 mM DETANONOate in water

Cultured cells of interest

Mito-HE

Warmed media

Dulbecco's phosphate buffered saline (PBS)

Confocal microscope equipped with:

Diode (UV) laser

Argon laser

- 1. Proceed with Basic Protocol 2, Steps 1 -6.
- 2. Dissolve 0.6 g of DETANONOate into 1 mL water for 3.68 mM solution. DETANONOate solutions can be frozen at -20°C for later use. The concentration of the DETANONOate solution should be checked using  $\varepsilon_{252}$ =7640  $M^{-1}$ cm<sup>-1</sup>.
- Add 24.5 µL of 3.68 mM DETANONOate to 3 mLs of media containing cells of interest
- 4. Incubate 20 min at 37°C.
- 5. Continue with Basic Protocol 2, Steps 7 12.

# BASIC PROTOCOL 3.

# Imaging live cell superoxide generation using wide field microscopy

Analysis of Mito-HE oxidation and superoxide generation can be readily applied to wide field microscopy as well as confocal microscopy.

# Materials

Cultured cells of interest

Mito-HE

Warmed Hank's balanced salt solution (containing calcium) or other appropriate live cell buffer

Epifluorescence microscope equipped with appropriate filters:

Example: 387 +/- 5.5 nm and 484 +/- 5.0 nm excitation filters and 580 nm +/-30 nm emission filters

- 1. Turn on arc lamp and microscope.
- Set up for live cell imaging using a heated stage with incubation chamber (37°C) and 5% CO<sub>2</sub> if bicarbonate-based buffer is used.
- To control for autofluorescence, obtain images of unlabeled cells using both excitation wavelengths, utilizing the longest exposure time and other image acquisition settings equal to those used for labeled cells (below).
- 4. Prepare Mito-HE solution by dissolving 50 μg of Mito-HE in 13 μL of DMSO for a 5 mM solution. Dilute 1 μL of the 5 mM solution into 10 μL buffer (final volume) for a 500 μM solution. Dilute this 500 μM solution by adding 2 μL per mL of labeling solution for cells to produce a 1 μM Mito-HE solution. *The optimal concentration of Mito-HE will vary with cell type and should be determined empirically; see Critical Parameters and Troubleshooting.*
- Gently rinse the cells twice with warm buffer and add Mito-HE solution to submerge cells for 15-20 min at 37°C which may be performed on a climate-controlled microscope stage or in a separate incubator.
- 6. If treatment of cells is required, obtain pre-treatment images at this point using both excitation wavelengths, minimizing

exposure time and number of image acquisitions over the time course to avoid photobleach and photo-oxidation effects.

- 7. Image cells with both 387 and 484 nm excitation for Mito-HE oxidation and superoxide oxidation.
- After imaging Mito-HE, it is possible to continue with live cell imaging of the mitochondria and nuclei (Support protocol 2) or with immunohistochemistry performed directly on the microscope (Support protocol 3).

#### **REAGENTS AND SOLUTIONS**

#### **Respiration buffer**

225 mM mannitol
75 mM sucrose
10 mM KCI
10 mM Tris HCI
5 mM KH<sub>2</sub>PO<sub>4</sub>
0.1% fatty acid free bovine serum albumin, pH = 7.2.

#### Xanthine Oxidase Suspension

Xanthine oxidase is typically purchased as a suspension in buttermilk. Centrifuge 250  $\mu$ L of the milk suspension at 2500 rpm for ~ 5 min. Discard supernatant and resuspend the pellet in 50 mM potassium phosphate by a quick vortex.

#### 5.3 COMMENTARY

#### **Background Information**

The discovery of superoxide dismutase by Fridovich and McCord would begin a revolution for the implications of biologically relevant free radicals, beginning with superoxide. Over the past 40 years superoxide has been implicated in nearly every pathological disorder and the mitochondria have become the center focus for superoxide production. A method to detect mitochondriallygenerated superoxide would be invaluable.

The problem lies in that methods assessing  $O_2^{\bullet}$  generation lack the sensitivity to compete with intracellular superoxide dismutase (SOD) and the selectivity for  $O_2^{\bullet}$  as compared to other oxidants. Mito-HE, a fluorescent superoxide detector targeted to the mitochondria, is oxidized by  $O_2^{\bullet}$  in a manner similar to HE, including an initial one electron oxidation to the Mito-HE radical (Scheme 2):

$$Mito-HE \xrightarrow{-1e^{-}} Mito-Et^{+}$$

$$Mito-HE \xrightarrow{+} Mito-HE \xrightarrow{-1e^{-}} Mito-Et^{+}$$

$$O_{2}^{+-} HO-Mito-Et^{+}$$

Although HE and Mito-HE react with  $O_2^{-}$  with a rate constant on the order of  $10^6 \text{ M}^{-1}\text{s}^{-1}$ , the second step of oxidation involves radical-radical coupling of  $O_2^{-}$  to the ethidium radical and will occur close to the diffusion limit  $(10^9 \text{ M}^{-1}\text{s}^{-1})$  making Mito-HE an efficient competitor of SOD (121). However, selective detection of HO-Mito-Et<sup>+</sup> is required. Conventional methods using 500 nm excitation induces fluorescence emission from both the hydroxlated and ethidium products due to their spectral overlap (66, 121). However, excitation at 396 nm enhances the fluorescence emission of HO-Mito-Et<sup>+</sup> by 70%, and reduces the spectral overlap of Mito-HE with confocal microscopy as excitation at 396 nm can result in increased autofluorescence making it difficult to see the signal from HO-Mito-Et<sup>+</sup>.

Superoxide detection is difficult because the assays currently available are susceptible to many potential artifacts. Rigorous methods such as a dualwavelength excitation method could help to unravel the role of superoxide in pathology and toxicological insults to the mitochondria.

#### Critical parameters and Troubleshooting

# The detection of superoxide from isolated mitochondria (Basic Protocol 1).

Time is a critical parameter in these experiments as the mitochondria can begin to uncouple after isolation. Uncoupling will become apparent as the mitochondria will fail to generate a superoxide inducible signal.

Mitochondrial superoxide generation will also vary greatly depending on how well the mitochondria remain coupled after isolation. Measuring oxygen consumption of the isolated mitochondria can provide invaluable information with regard to interpreting superoxide generation. It is useful to measure superoxide generation and convert that to terms of percent of electron leakage by dividing by the oxygen consumption. Mitochondria with lower oxygen consumption may inherently generate less superoxide. Oxygen consumption measurements should be determined at the same time as the superoxide experiments to use time most efficiently before the mitochondria uncouple. Ideally, after isolation the mitochondrial suspension should be split into two aliquots for assessment of oxygen consumption and superoxide detection on the fluorometer, and this experiment may require more than one investigator.

Mito-HE is susceptible to photooxidation to the ethidium analog, Mito-Et<sup>+</sup>. Although 396 nm excitation decreases spectral overlap to 10%, Mito-Et<sup>+</sup> is still fluorescent at 396 nm excitation. Photooxidation can generate a significant quantity of Mito-Et<sup>+</sup> thereby increasing fluorescence emission not only at 510 nm excitation but also at 396 nm excitation. If this is the case, the rate at 510 nm excitation should be much more rapid than the rate observed using 396 nm excitation, indicative of Mito-Et<sup>+</sup> generation. In addition, 396 nm excitation may induce superoxide formation from molecular oxygen in buffers, and thereby generate the superoxide product and increase fluorescenct emission using 396 nm excitation. Therefore Mito-HE concentrations should be kept as low as possible (i.e. 0.8  $\mu$ M) and the fluorescent signal from Mito-HE alone should be obtained as a fluorescent control.

Moreover, dilute Mito-HE solutions can autooxidize in buffer alone, presumably reacting with oxygen and thereby generating increasing amounts of Mito-Et<sup>+</sup>. Dilute Mito-HE solutions in potassium phosphate buffer should be replaced at least every two hours.

The concentration of mitochondrial protein should be kept within a range close to 1 mg/ mL. If the changes in fluorescence are modest, increasing mitochondrial concentration may enhance the superoxide signal. However it is also possible at higher concentrations (> 1 mg/mL) for the mitochondria to consume all of the available oxygen, causing a decrease in membrane potential and release of Mito-HE. If higher concentrations of mitochondria are desired, anaerobiosis can be prevented by a fine flow of oxygen onto the surface of the suspension.

Antimycin is diluted into ethanol and therefore a control from the effects of ethanol alone should be performed.

# The detection of superoxide in live cells using confocal and wide field microscopy (Basic Protocols 2 and 3).

Autofluorescence emitting from both 405 and 514 nm excitation can be intense in some cell types, therefore collecting autoflurescent images from untreated cells prior to assaying for superoxide is important.

If no difference in fluorescent intensity is observed after incubation with Mito-HE, several parameters can be considered. It may be necessary to image cells at a later time point, more than one hour, to detect fluorescence. The concentration of Mito-HE is critical and cell type specific. In our experience, the optimal concentration of Mito-HE varies from  $0.1 - 1 \mu M$ . Mito-HE will accumulate within the mitochondria approximately one thousand fold, and therefore a 1  $\mu$ M incubation with Mito-HE can result in 1 mM within the mitochondria. High concentrations of Mito-HE can be toxic, causing the mitochondria to rupture. We have observed a rapid loss of fluorescence from mitochondria and subsequent redistribution to the nucleus after incubation with as little as 2  $\mu$ M Mito-HE. Nuclear staining may indicate mitochondrial toxicity

due to high concentrations of Mito-HE. Excessive Mito-HE accumulation may also stress mitochondria and enhance their sensitivity to inhibitors or other molecules targeted to the mitochondria. Because Mito-HE accumulation depends upon mitochondrial membrane potential ( $\Delta \psi$ ), cells with compromised  $\Delta \psi$  will accumulate Mito-HE to a lesser extent, which can lead to under-estimating superoxide generation. It may be necessary to measure  $\Delta \psi$  when observing differences in superoxide between two different cellular conditions. Because DNA is necessary to enhance the fluorescence of oxidized Mito-HE, fluorescence might be limited by the amount of mitochondrial DNA, particularly in cells depleted of mitochondrial DNA (rhonaught cells).

Fluorescence intensity could artificially increase due to photooxidation. Photooxidation may be minimized by using the lowest possible concentration of Mito-HE and reducing the exposure to light throughout an experiment. A minimum number of images should be taken, for example at beginning and end time points, or every 15 minutes between.

It is also important to consider the condition of the cells under investigation, such as the percent of serum in the media. Placing cultured astrocytes on low serum (2% horse serum) allows the cells to take on a more stable and differentiated phenotype. We were able to detect superoxide generation from astrocytes maintained in media containing 2% horse serum, which was not detectable when cells were maintained with 10% fetal bovine serum. The serum and phenol red in media do not appear to affect fluorescent detection of Mito-HE oxidation. Confluency and age of cells are also variables in the experiment that should be accounted for.

#### Identification of mitochondria and nuclei (Support Protocol 2)

Mito-HE can dissipate the membrane potential and thereby decrease the uptake of other mitotracker probes that depend upon the membrane potential. The optimal concentration of Mitotracker Deep Red varies with cell type and should be kept as low as possible to decrease potential artifacts from mitochondrial overload and toxicity.

#### Immunohistochemistry following Live Cell Imaging (Support Protocol 3)

One difficulty with immunohistochemistry is the limitation of available wavelengths because detection of Mito-HE is utilizing both 405 nm and 514 nm excitation. DAPI is a remarkably intense dye and we have found that instrument parameters for the detector can be decreased so as to obtain nuclear staining from DAPI using 405 nm excitation without observing fluorescence from Mito-HE. We have used secondary antibodies conjugated to the fluorescent dye Cy5 which utilizes 633 nm excitation from a HeNe2 laser and does not interfere with Mito-HE fluorescence. Signal bleeding is a potential problem and should be assessed for example by collecting fluorescent emission from Mito-HE using the Cy5 settings. It is also important to check for non specific binding by assaying fluorescence that is obtained from incubation with the secondary antibody only, omitting the primary antibody.

Immunohistochemistry can be used to identify different cells which have been cocultured together and analyzed for superoxide. Cell tracker probes are also available, which can be added to cells before mixing with other cell types. However we have found several of the cell tracker dyes utilize antioxidants within the cell subsequent to their internalization. Therefore cell tracker dyes can artificially increase superoxide generation due to the decrease in intracellular antioxidant levels.

#### **Anticipated Results**

# The detection of superoxide from isolated mitochondria (Basic Protocol 1).

Typical traces of superoxide generation from isolated mitochondria stimulated with antimycin are presented in Figure 5.1. Prior to antimycin addition, a basal level of superoxide production can be observed using both

396 nm excitation (Figure 5.1a) and 510 nm excitation (Figure 5.1b). After antimycin addition, the rate of fluorescence emitted was higher using 510 nm excitation (Fig. 5.1d) than at 396 nm excitation (Figure 5.1c). The rate of fluorescence generation from antimycin-stimulated mitochondria was 31% greater at 510 nm versus 396 nm excitation, suggesting that Mito-Et<sup>+</sup> was being formed by non-superoxide dependent pathways and  $O_2^{-}$  formation was overestimated using 510 nm excitation.

After equilibriating reagents and testing fluorescence due to the incubation of Mito-HE alone, the addition of mitochondria will significantly increase the level of fluorescence emission due to light scattering from the mitochondrial suspension. Increases in fluorescence can continue in either a linear or exponential fashion and the calculated rate should be obtained by fitting the data curve appropriately.

Selectivity for superoxide can be shown by inhibiting the fluorescent signal by pre-incubating with a superoxide scavenger such as nitric oxide or adding superoxide dismutase.

# The detection of superoxide in live cells using confocal and wide field microscopy (Basic Protocols 2 and 3).

Excitation at 405 nm is selective for the superoxide product whereas 514 nm excitation induces fluorescence emission from both the superoxide product and Mito-Et<sup>+</sup>. However, different information is obtained from excitation at 405 and at 514 nm. In some cell types, autofluorescence at 405 nm can be so intense as to make the superoxide signal seem diffuse or difficult to observe, due to the intensity of perinuclear autofluorescence (Figure 5.2a).

On the other hand, intense staining from 514 nm excitation that does not co-localize with fluorescent emission from 405 nm excitation indicates oxidation of Mito-HE by non-superoxide dependent processes (Figure 5.2b; right center). Figure 5.2b also shows an intensely autofluorescent cell, as indicated by fluorescence emitting from 405 nm excitation and not 514 nm excitation (Figure 5.2b; bottom center). Superoxide generation will produce colocalized fluorescence, merged from both 405 nm and 514 nm excitation that is reticular and mitochondrial-like (Figure 5.2c).

The detection of superoxide using Mito-HE will be a generally semiquantitive assay for intracellular  $O_2^{\bullet}$  because the relative fraction of  $O_2^{\bullet}$  reacting with SOD will be unknown. A second confounding variable can be the endogenous production of nitric oxide competing for  $O_2^{\bullet}$ , which may need to be inhibited by nitric oxide synthase inhibitors for  $O_2^{\bullet}$  to be detectable. Exact quantitation of products using fluorescence microscopy is fraught with difficulties. HPLC methods are available to quantify relative amount of hydroxylated and non-hydroxylated HE when testing for cytosolic superoxide detection (15, 16). However, the extraction methods for hydroethidine oxidation products. Mito-HE oxidation products tend to result in a partition between the organic and non-organic phase, most likely due to the increased hydrophilicity from the positive charge on the triphenylphosphonium group.

If intracellular oxidation of Mito-HE to Mito-Et<sup>+</sup> should become more than 10-20 fold greater than HO-Mito-Et<sup>+</sup>, a majority of fluorescence from 396 nm excitation could be due Mito-Et<sup>+</sup>. However, monitoring the oxidation at both 396 and 510 nm will reveal this potential artifact.

#### **Time Considerations**

# The detection of superoxide from isolated mitochondria (Basic Protocol 1).

The assay described will typically take one full day, depending upon the time and method for preparing isolated mitochondria. It is important to move quickly and the mitochondria should be tested for superoxide generation and/or oxygen consumption immediately after isolation to avoid upcoupling of the mitochondria. In general, the mitochondria are likely to be uncoupled 3 -4 hours after isolation. Fluorometers reading a single cuvette, rather than a

plate reader, are more sensitive instruments however the experiments need to be performed in sequence rather than simultaneously as is accomplished with a plate reader. Therefore, sequential experiments which cover several experimental parameters and conditions, including excitation at 510 and 396 nm, will easily consume the 3 -4 hours while the mitochondria are viable. Data analysis from the fluorescent traces can be performed the following day.

# The detection of superoxide in live cells using confocal and wide field microscopy (Basic Protocols 2 and 3).

The time to complete superoxide detection in live cells will vary especially depending on the number of fields imaged. Experiments range from 4 -5 hours and the majority of one full day should be set aside. The confocal should be set up and equilibriated for live cell imaging at 37°C. In addition to the listed incubation times, placement of cells, focusing, image acquisition, especially high resolution images, add a significant amount of time to the assay. It is also necessary to proceed cautiously, so as to not move the stage or the dish containing cells while aspirating or adding reagents.

#### Identification of mitochondria and nuclei (Support Protocol 2)

Collecting the superoxide data will take the largest amount of the time at which point cells will be ready to image for mitochondria and nuclei. This should only add ~45 min to Basic Protocol 2, due to incubation times and imaging.

## Immunohistochemistry following Live Cell Imaging (Support Protocol 3)

Performing immunohistochemistry on cells assayed for superoxide detection requires an approximate 9 hour day, including time for Basic Protocol 1. Careful aspiration, addition of reagents, focusing, moving to several fields and obtaining high resolution images of those fields requires a significant amount of time, especially if several fields are to be examined. It is important to consider the time required if the confocal microscope being used is run by a facility which charges by the hour. If charged hourly, immunohistochemistry combined with live cell imaging can become a costly experiment.

In order to increase reproducibility, the scheduled time for incubations should be followed closely each time. This can be more readily accomplished by multi-tasking and preparing reagents for the following step during incubation times of the previous step, as most reagents should be used fresh.

The time required for the experiment may also increase due to different incubation times for the selected primary and secondary antibodies.

#### Acknowledgments

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### 5.4 Figure Legends

Figure 5.1 Typical fluorescent traces from isolated mitochondria incubated with Mito-HE. Rat liver mitochondria (600  $\mu$ g protein/mL) were incubated with 0.8  $\mu$ M Mito-HE and reagents for State III respiration (10 mM succinate, 8.25  $\mu$ M rotenone and 2.5 mM ADP) in respiration buffer. Fluorescence was monitored using 396 nm excitation (a) and 510 nm excitation (b). Superoxide generation was stimulated with the addition of 15  $\mu$ M antimycin (arrows) and the rate of fluorescence increase was 31% higher using 510 nm excitation (d) than 396 nm excitation (c).

Figure 5.2 Live astrocytes incubated with Mito-HE and imaged by confocal microscopy. The superoxide product, HO-Mito-Et<sup>+</sup>, is excitable using both 405 (blue) and 514 nm (red) excitation wavelengths. (A) Perinuclear autofluorescence can be more intense using 405 nm excitation, making it difficult to detect HO-Mito-Et<sup>+</sup>. (B) Fluorescence emitting from 514 nm excitation only can be an indicator of non-specific oxidation of Mito-HE (B, right center). (C) Superoxide generation is best detected from colocalized fluorescence, merged from both 405 nm and 514 nm excitation that is punctate and mitochondrial.

5.5 Figures
Figure 5.1





Figure 5.2 (b)

Figure 5.2 (a)

Figure 5.2 (c)

Chapter 6

The selective detection of increased superoxide generation in astrocytes expressing the ALS-associated SOD1<sup>G93A</sup> mutation

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## 6.1 Abstract

Astrocytes are thought to play a critical role in the death of motor neurons in amyotrophic lateral sclerosis (ALS). ALS is a debilitating disease involving progressive paralysis of voluntary muscles as a consequence of motor neuron degeneration. Although characterized by the death of motor neurons, ALS is not a cell-autonomous disease. Astrocytes isolated from the ALS model of SOD<sup>G93A</sup> over-expressing rats induce apoptosis in nontransgenic motor neurons when co-cultured. How SOD1<sup>G93A</sup> mutations confer toxicity is not fully understood. Protection mediated by elevated glutathione content suggests the involvement of oxidative damage. Mitochondria are dysfunctional in ALS and can be a major source of superoxide when disrupted. The detection of superoxide in mitochondria is experimentally challenging. In this work, we utilized a method we recently developed which increases the selectivity of detecting mitochondrially-generated superoxide using a derivative of hydroethidine. Indeed, SOD1<sup>G93A</sup> astrocytes have increased superoxide generation at the mitochondria, in spite of having paradoxically increased superoxide scavenging activity. The temporal accumulation of SOD1<sup>G93A</sup> with the mitochondria was required for superoxide generation to be observable. Increased superoxide production was inhibited not only by the superoxide scavenger, nitric oxide, but also by the copper chelator, tetrathiomolybdate, implying activation of superoxide generation required copper. SOD can be imported into the mitochondria and metallated to establish intermembrane space SOD. However, the gross over-expression of SOD<sup>G93A</sup> in the ALS rats may overwhelm mitochondrial capabilities to fully supply metals to SOD<sup>G93A</sup>. Copper replete, zinc deficient SOD<sup>G93A</sup> may be catalyzing superoxide formation, and in turn damaging respiratory chain complexes, compounding superoxide production. This work provides evidence that mitochondrial dysfunction and increased oxidant production may be important mechanisms in the toxicity of mutant SOD1<sup>G93A</sup> expression in familial ALS.

#### **6.2 Introduction**

Astrocytes represent the largest cell population in the central nervous system. Although once viewed simply as the glue of the central nervous system (CNS)(73), astrocytes have, in the past decade, been found to interact closely with neurons to provide structural, metabolic and trophic support, and actively participate in modulating neuronal excitability and neurotransmission by controlling the extracellular levels of ions and neurotransmitters (123). Thus, it has been proposed that the selective and rapid loss of neurons within neurodegenerative disorders may be largely modulated by astrocytes (74, 124).

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the progressive loss of motor neurons in the ventral horn of the spinal cord, brain stem, and motor cortex (125). Mutations to Cu-Zn superoxide dismutase (SOD1) are a well known cause of familial ALS, occurring in only ~2% of ALS patients (1). Mice which over-express ALS-linked mutated SOD1 selectively lose motor neurons, developing a pathology similar to human ALS and are the most widely used model of the disease (2). Although ALS involves the selective death of motor neurons, evidence indicates that ALS is not a cell autonomous disease and the onset and progression of the disease is largely affected by non-neuronal cells (75, 76, 126).

A number of reports support the critical role of astrocytes in ALS pathogenesis (75, 76). For example, over-expression of the ALS-associated mutation, SOD1<sup>G37R</sup>, in mice results in an aggressive form of ALS with extensive reactive astrocytosis. However, selective expression of SOD1<sup>G37R</sup> in neurons only is not sufficient to induce motor neuron disease (75). Knocking down the expression of mutant SOD1 in the glia of chimeric mice delays ALS disease progression (76). A strong glial reaction typically surrounds both upper and lower motor neurons in ALS patients and animal

models of the disease (74). In animal models, astrogliosis becomes evident before the onset of motor symptoms and dramatically increases with the progression of the disease (127-129). Moreover, astrocytes isolated from the spinal cord of SOD1<sup>G93A</sup> rats can induce apoptosis of co-cultured motor neurons (31).

How the SOD1<sup>G93A</sup> mutation confers the ability of astrocytes to become lethal to motor neurons is not fully understood. Increasing glutathione levels via *t*-butylhydroquinone activation of Nrf-2 prevents motor neuron apoptosis induced by SOD1<sup>G93A</sup> astrocytes (31). This result implies reactive oxygen or nitrogen species may be important in mediating neuronal death. Indeed, SOD1<sup>G93A</sup> astrocytes display increased production of nitric oxide (31) and nitric oxide can quickly react with superoxide to generate peroxynitrite. Peroxynitrite can also induce astrocyte activation, promoting the development of long processes and increasing immunoreactivity for GFAP, nitric oxide synthase, and nitrotyrosine (130). Notably, astrocytes that display this phenotype are cytotoxic to motor neurons in culture, and furthermore, are also found throughout the spinal cords of SOD1<sup>G93A</sup> mice. The increase in nitric oxide in SOD1<sup>G93A</sup> astrocytes and observed changes phenotypic and indicative of peroxynitrite generation imply that superoxide generation may be increased in astrocytes as well.

Superoxide is thought to be generated largely from electrons leaking from the respiratory chain of the mitochondria. A growing body of evidence suggests mitochondria are dysfunctional in ALS (125, 129, 131). The mitochondria of SOD1<sup>G93A</sup> mice become large and vacuolated at onset of disease (129). Similar vacuoles and "giant" mitochondria and dense aggregated mitochondria have been observed in degenerating motor neurons in ALS patients (125). Notably, ALS mutant SOD1s have been observed to associate with spinal cord mitochondria but not with brain or muscle mitochondria (77). Evidence supports mutant SOD1s reside within the intermembrane space, the matrix and as integral membrane components on

the cytoplasmic face of the mitochondria (77). Toxicity from mitochondrial mutant SOD1 may be a result of mutant SOD1 association with Bcl-2 and subsequent inhibition of its anti-apoptotic activity (132). In motor neuronal cell lines, mutant SOD1 expression causes mitochondrial morphological alterations (78) and elicits a shift in mitochondrial redox state (79). Because superoxide generation is thought to occur largely within the mitochondria, we sought to investigate if SOD1<sup>G93A</sup> over-expression within astrocytes causes mitochondria dysfunction through increased superoxide generation.

Assessing the generation of superoxide can be difficult and confounded with the assays currently available (121). Chemiluminescent probes can actually generate  $O_2^{-}$  by either uncoupling the respiratory chain or reacting with oxygen (101, 102). Fluorescent probes, such as the reduced forms of fluorescein, rhodamine and ethidium, are susceptible to autooxidation through radical intermediates when illuminated (57) and can also be oxidized by a variety of intracellular peroxidases, oxidases or cytochromes to yield radical intermediates that dismutate to give fluorescent products (53, 54, 56, 65). Hydroethidine (HE), the reduced form of ethidium (Et<sup>+</sup>), is currently the most popular fluorescent probe used for superoxide detection. The accepted practice of 510 nm excitation does not distinguish between the unspecific, intracellular oxidation of HE to Et<sup>+</sup>, and the selective oxidation of HE to the superoxide product, Hydroxy-ethidium (HO-Et<sup>+</sup>) (66, 67). However, HO-Et<sup>+</sup> has a selective excitation wavelength at 396 nm which enhances fluorescence emission 70% and decreases the spectral overlap with  $Et^+$  to 10% (121). The mitochondrially targeted derivative of HE, Mito-HE (MitoSOX Red<sup>™</sup>, Invitrogen, Inc.) is also selectively oxidized by  $O_2^{\bullet}$  to a hydroxylated product which is selectively excited at 396 nm (121). We utilized the improved method of combining 400 and 500 nm excitation to monitor Mito-HE oxidation to better assess the question of SOD1<sup>G93A</sup> involvement with mitochondria and increased superoxide generation.

## 6.3 Results

Superoxide generation was increased in astrocytes over-expressing SOD1<sup>G93A</sup> as compared to their neighboring, non-transgenic cells (Fig. 6.1 A-Co-culturing SOD1<sup>G93A</sup> transgenic astrocytes with non-transgenic E). astrocytes allowed for a direct comparison of transgenic and non-transgenic cells under identical experimental conditions (Fig. 6.1). This method of fluorescence analysis is advantageous because it eliminates inter-sample discrepancies that may arise due to instrument settings or culture conditions. Fluorescence emitting from both 405 nm excitation (green) and 514 nm excitation (red) confirms O2<sup>-</sup> oxidation of the fluorescent probe, Mito-HE (merged, yellow). Because the copper in SOD1 has been shown to catalyze oxidant generation (133) and damage (134), the copper chelator, tetrathiomolybdate (TTM) was added to astrocyte cultures. Indeed, TTM inhibited superoxide generation from SOD1<sup>G93A</sup> transgenic astrocytes (Fig. 6.1 F-J). As a control, a small flux of nitric oxide from DETANONOate was added to cultures to scavenge superoxide. DETANONOate also inhibited Mito-HE oxidation in SOD1<sup>G93A</sup> astrocytes (Fig. 6.1 K-O). DETANONOate will induce the formation of peroxynitrite in the presence of superoxide and peroxynitriteoxidized Mito-HE is not excited using 405 or 514 nm. Moreover, DETANONOate is advantageous to other superoxide scavengers such as MnTBAP, because MnTBAP can confound experiments that utilize HE. A difficulty is that MnTBAP can react directly with HE to generate Et<sup>+</sup> (122). In addition, MnTBAP absorbs light at 470 nm which can be used to detect fluorescence from the hydroxy-product (122). Quantification of fluorescent cells revealed that 82% of cells positive for SOD1<sup>G93A</sup> also displayed increased fluorescence for superoxide generation (Fig. 6.2). The addition of TTM reduced the number of SOD1<sup>G93A</sup> astrocytes displaying increased superoxide generation to 15% and DETANONOate reduced this value to 33% (Fig. 6.2).

Both 405 and 514 nm excitation were used to detect superoxide generation. The superoxide product of Mito-HE, the hydroxylated product

 $(HO-Mito-Et^{+})$  is excitable using both 405 and 514 nm excitation (121). However, the two electron oxidation product, Mito-Et<sup>+</sup>, which can be an indicator of non-specific intracellular oxidation of Mito-HE, emits fluorescence when excited at 514 nm. On the other hand, 405 nm-excitable autofluorescence can be so intense that the superoxide signal appears diffuse or is difficult to observe due to the intensity of perinuclear autofluorescence.

Interestingly, fluorescence from over-expressed SOD1<sup>G93A</sup> colocalized with fluorescence from the mitochondrial targeted probe, Mito-HE (Fig. 6.1) indicating SOD1<sup>G93A</sup> associates with the mitochondria. Moreover, in cocultures analyzed at an earlier time point (24 versus 32 days since isolation), two different patterns of SOD1<sup>G93A</sup> expression was observed: 36% of SOD1<sup>G93A</sup> astrocytes displayed a diffuse cytosolic immunoreactivity for SOD1 over-expression (Fig. 6.3 A) while 64% of SOD1<sup>G93A</sup> astrocytes showed a more punctate immunoreactivity, indicative of mitochondrial localization (Fig. 6.3 B, see also Fig. 6.1). Interestingly, transgenic cells with the cytosolic diffuse staining for SOD1<sup>G93A</sup> did not show increased HO-Mito-Et<sup>+</sup>, superoxide-dependent fluorescence (Fig. 6.3, 1-10), as compared to transgenic cells with mitochondrial SOD1<sup>G93A</sup> localization (Fig. 6.3, 11-20). This result suggests that the increased superoxide generation from the mitochondria correlates with the accumulation and association of SOD1<sup>G93A</sup> with the mitochondria.

To determine if the increase in superoxide generation could be attributed to a decrease in superoxide dismutating activity in SOD1<sup>G93A</sup> astrocytes, astrocytes were permeabilized and assayed for SOD1 activity. Superoxide scavenging activity was increased in SOD1<sup>G93A</sup> astrocytes as compared to non-transgenics (Fig. 6.4).

## 6.4 Discussion

Paradoxically, astrocytes that over-express SOD1<sup>G93A</sup> have increased superoxide generation and also have increased superoxide scavenging activity. Increased superoxide generation also appeared to be dependent

upon the temporal accumulation of SOD1<sup>G93A</sup> with the mitochondria. How the association of SOD1<sup>G93A</sup> with the mitochondria increases ROS production is not fully understood. There are at least two plausible mechanisms, which may be acting synergistically to increase superoxide. First, the association of SOD1<sup>G93A</sup> with the mitochondria may cause aberrant oxidative phosphorylation and thereby facilitate increased superoxide production. Second, accumulation of copper-replete, zinc-deficient SOD1 within the mitochondria may be generating superoxide: newly synthesized, apoSOD1 with the cysteine bridge between C57 & C146 reduced can be transferred into the innermembrane space of the mitochondria (135). The copper chaperone, CCS, metallates SOD1 with copper after zinc insertion to establish innermembrane CuZnSOD1 (135). Increased over-expression of SOD1<sup>G93A</sup> overwhelm intramitochondrial capabilities fully metallate may to innermembrane space SOD1, resulting in the accumulation of partially metallated SOD1s within the innermembrane space (IMS). Cu-replete zincdeficient SOD1 can catalyze the formation of peroxynitrite in the presence of nitric oxide presumably by generating superoxide (133). Thus, zinc deficient SOD1 within the IMS may itself be the species increasing superoxide production at the mitochondria. The inhibition of superoxide generation by the copper chelator, TTM, supports a copper-dependent mechanism of oxidant Increased superoxide generation from partially metallated, generation. copper-containing, zinc-deficient SOD1 within the mitochondria could in turn damage respiratory chain complexes, compounding superoxide production from both zinc deficient SOD1 and damaged respiratory chain complexes.

Alternative explanations on how TTM might be inhibiting superoxide generation are problematic. Free copper can catalyze the formation of ROS, however the high demand of copper due to the gross over-expression of SOD1 in transgenic mice should leave little to no free copper. If TTM was chelating copper within cytochrome *c* oxidase and thereby affecting respiratory chain activity, one would expect an increase in ROS. Decreases in copper

availability have been shown to decrease the activity of cytochrome *c* oxidase causing mitochondrial dysfunction and increased ROS (136).

Increased superoxide generation has also been reported in SOD1<sup>G93A</sup> microglia, generated from NADPH oxidase (137). It is interesting that the two principal sources in vivo for superoxide generation, NADPH oxidase and the mitochondria, have been proposed to be linked and work in conjunction (138). Increased superoxide generation from the mitochondria in human 293T cells stimulates superoxide production from NADPH oxidase (138). Signalling from increased mitochondrial superoxide generation has been proposed to stimulate NADPH oxidase to compound ROS production and induce cell death (138). Increased mitochondrial superoxide generation in astrocytes may be triggering NADPH oxidase and subsequent superoxide generation in neighboring cells, such as microglia and motor neurons.

Assessing superoxide generation is experimentally difficult due to the lack of a probe that selectively reacts with superoxide and competes with intracellular SOD1. The finding that the superoxide product of Mito-HE can be excited at 396 nm, an excitation that is not present for the non-specific oxidation product, increased the selectivity of using Mito-HE for measuring superoxide (121). In addition, the mechanism of superoxide oxidation of Mito-HE indicates the second step of the reaction involves radical-radical coupling of superoxide to the Mito-HE radical, occurring at the diffusion limit and efficient creating competitor of SOD1 (121). an Moreover. triphenylphosphonium groups strategically place compounds within the mitochondria (71). Triphenylphosphonium derivatives will cross mitochondrial membranes and lodge themselves within potential energy wells residing on the edges of membranes (71). The triphenylphosphonium group will lie just within the negatively charged matrix, leaving the hydrophobic moiety within the membrane (71). This strategic placement of Mito-HE may facilitate the preferential ability of Mito-HE to trap superoxide generated from membrane components, before the diffusion of superoxide to react with cytosolic

SOD1<sup>G93A</sup>. This may explain how increased superoxide generation is detectable in cells with increased, cytosolic, superoxide scavenging activity.

These results support a direct role for SOD1<sup>G93A</sup> increasing ROS production at the mitochondria. Increased ROS production may damage mitochondrial respiratory chain complexes, compound ROS production, and damage mitochondrial DNA. Accordingly, increased oxidative damage to mitochondrial proteins and lipids has been observed in SOD1<sup>G93A</sup> mice (139). SOD1<sup>G93A</sup> accumulation within mitochondria may also interfere with chaperone activity to import proteins to the mitochondria which are essential for normal function (129). Mitochondrial dysfunction can cause an energy deficiency and ionic imbalance, which could increase intracellular vulnerability to excitotoxicity, oxidative stress, and the release of pro-apoptotic proteins (129). Our results support a role for mutant SOD1 expression conferring increased superoxide generation and imply mitochondrial dysfunction as a result of mutant SOD1 expression. Thus, antioxidants targeted to the mitochondria may be therapeutic in familial ALS.

# 6.5 Materials and Methods

MitoSOX Red<sup>™</sup>, DAPI (4',6-diamidino-2-phenylindole), Mitotracker Deep Red<sup>™</sup>, and AlexaFluor<sup>™</sup> 532 goat anti-rabbit IgG were obtained from Invitrogen-Molecular Probes, Inc. Unless otherwise specified, all other reagents were from Sigma (St. Louis, MO, USA). Culture media and serum were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). Rabbit anti-human SOD antibodies were obtained from Cell Signaling Technology, Inc. Sprague-Dawley SOD1<sup>G93A</sup> L26H rats were kindly provided by Dr. David S. Howland (Wyeth Research, Princeton, NJ) (26).

Primary astrocyte cultures were prepared from 1 day old Sprague-Dawley rat pups according to the procedures of Saneto and De vellis with minor modifications (130). Transgenic SOD1<sup>G93A</sup> and non-transgenic pups were genotyped by PCR. SOD<sup>G93A</sup> transgenic astrocytes were cultured

separately from non-transgenic astrocytes. Astrocytes were plated at a density of 2 X 10<sup>4</sup> cells/cm<sup>2</sup> and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, HEPES (3.6 g/L), penicillin (100 IU/mL) and streptomycin (100 µg/mL). After initial growth conditions, shaking, and cytosine treatment, astrocyte cultures were grown to confluency. Confluent cultures were trypsinized with 0.025% trypsin and 0.02% EDTA in PBS for five minutes, collected in a 50 mL Falcon tube and centrifuged at 1000 rpm for 10 min. Cells were counted, and then SOD<sup>G93A</sup> transgenic and non transgenic were mixed at an approximate 1:1 ratio. Approximately 37,000 cells suspended in 250 µL droplet were placed on the center glass of a Mat Tek 35 mm glass bottom microwell dish (uncoated, Mat Tek Corporation). Cells were allowed to attach for ~2 hrs before adding a final volume of 3 mLs of media. This process allowed the maximal number of astrocytes to be centered and grown on the inner glass portion of the dish for analysis. Astrocyte cultures were grown to confluency before assessing superoxide production (3-7 days).

Astrocytes were placed on a low serum (0.5% fetal bovine serum) media ~48 h prior to superoxide assessment. Cultured cells were transferred to the heated stage (37°C) of a Zeiss LSM510 confocal microscope with a humidified atmosphere of 5% CO<sub>2</sub>. Live cells were imaged with a 63X oil immersion objective using either 405 nm or 514 nm laser excitation. The coordinates for several fields (6 – 8) were marked in order to follow specific fields throughout an entire experiment. Prior to Mito-HE incubation, each field was imaged for autofluorescence using both 405 and 514 nm excitation. Some cells are extremely autofluorescent, most likely related to the age of the astrocytes, and these fields were not used because of difficulties in detecting changes in Mito-HE fluorescence. The detector gain and amplifier offset values were adjusted to minimize autofluorescence, and then held constant for Mito-HE imaging.

Mito-HE was freshly prepared immediately after autofluorescent imaging by dissolving Mito-HE in DMSO for a 3.96 mM solution. Then, Mito-HE was diluted to 396  $\mu$ M in PBS and 2.27  $\mu$ L of the 396  $\mu$ M solution was added to the cells on the microscope (suspended in 3 mLs of media) for a final concentration of 0.3  $\mu$ M Mito-HE. Cells were incubated with 0.3  $\mu$ M Mito-HE for 20 min at 37°C (5% CO<sub>2</sub>) on the stage of the microscope. The media was then aspirated and replaced, and cells were incubated for an additional 40 minutes. Images were then obtained using the 405 and 514 nm excitation. Alternatively, prior to Mito-HE incubation, astrocytes were incubated with 30  $\mu$ M DETANOnoate. To determine the involvement of copper, astrocytes were either incubated with 1 nM tetrathiomolybdate (TTM) 24 hrs prior to Mito-HE imaging, or with 100 nM TTM 1 hr prior to Mito-HE imaging.

After Mito-HE imaging, immunohistochemistry was performed directly on the stage of the confocal microscope (kept at 37°C) to determine which astrocytes were transgenic for SOD1<sup>G93A</sup>. The media was aspirated and freshly diluted 4% formaldehyde in PBS was added to the cells on the microscope stage and incubated for 15 min. The fixative was aspirated and cells were washed gently with three, five minute PBS incubations. Cells were then permeabilized with 0.3% TritonX 100 for 10 minutes. Cells were blocked using 10% goat serum, 2% bovine serum album, 0.1% Triton X 100 in PBS for 50 min. The primary antibody for human SOD (Cell Signaling Technology, Inc), diluted 1/100 into PBS, was added for 60 min and cells were then gently washed with two, 5 min incubations with PBS. The secondary antibody AlexaFluor<sup>™</sup> 532 goat anti-rabbit IgG, diluted 1/100, was added for 60 min, and then the cells were again washed with two, 5 min incubations with PBS, and resuspended in 2 mLs of PBS. 0.8 µL of a 1 mg/mL DAPI solution was added to cells in PBS. Cells were imaged for SOD and DAPI using the 543 nm line of an argon laser and the 405 nm line of a diode laser.

Increased superoxide assayed by Mito-HE, inhibition with DETANOnoate and inhibition with TTM were quantified using all fields imaged

from three different astrocyte culture preparations. Cells emitting intense fluorescence for SOD as compared to the neighboring cells (visualized with DAPI) were counted as SOD1<sup>G93A</sup> positive. Cells emitting an intense, punctate-mitochondrial like fluorescence from both 405 and 514 nm excitation were counted as Mito-HE positive for increased superoxide generation.

To assess the membrane potential, SOD1<sup>G93A</sup> and non-transgenic astrocytes were kept cultured separately and then incubated with 3.5 nM Mitotracker Deep Red<sup>TM</sup> for 25 min and 0.8  $\mu$ L of a 1 mg/mL DAPI for 10 min. Live cell imaging was performed on the heated stage (37°C) of a Zeiss LSM510 confocal microscope with constant 5% CO<sub>2</sub>.

For analysis of SOD activity, astrocytes were harvested, and cells were lysed by repeated freeze-thawing. Supernatants were then collected, and protein concentrations determined by the method of Bradford (140). Non-denaturing gel electrophoresis was performed using a 12% polyacrylamide gel (pH 8.0) with a 5% stacking gel (pH 6.7) 1 to 12 mg protein was loaded in each well. Following electrophoresis, the gel was subject to nitro blue tetrazolium/riboflavin staining as described by Beauchamp and Fridovich (141). Briefly, The gel was first soaked in 25 mL of 1.23 mM NBT for 15 min, briefly washed, then soaked in the dark in 30 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 2.8 mM riboflavin for another 15 min. The gel was briefly washed again, and then illuminated and visualized on a light box. All the procedures were carried out at room temperature.

# 6.6 Figure Legends.

Figure 6.1 Superoxide generation is increased in SOD1<sup>G93A</sup> astrocytes. Superoxide generation was assessed by detecting colocalized fluorescence (yellow, merged) from two excitation wavelengths: 405 nm (green) and 514 nm (red). After live cell imaging, immunohistochemistry was performed to identify which of all cells (nuclei turquoise with DAPI) were SOD1<sup>G93A</sup> transgenic (purple). Astrocytes transgenic for SOD1<sup>G93A</sup> (purple, D) also showed increased fluorescence for the superoxide product, HO-Mito-Et<sup>+</sup> (A-C), as observed in the final merged view (E). The copper chelator, tetrathiomolybdate (TTM), inhibited increased superoxide generation from SOD1<sup>G93A</sup> astrocytes (F-J). DETANONOate (30  $\mu$ M), a nitric oxide generator, was used as a superoxide scavenger and control, and inhibited superoxide generation in SOD1<sup>G93A</sup> astrocytes (K-O).

Figure 6.2 Quantification of increased superoxide generation in SOD1<sup>G93A</sup> astrocytes. Eighty-two percent of SOD1<sup>G93A</sup> astrocytes displayed increases in superoxide generation (as assessed by oxidized Mito-HE fluorescence). Only 11% of non-transgenic cells showed increases in superoxide generation. The addition of TTM decreased the number of SOD1<sup>G93A</sup> astrocytes with increased superoxide to 15%. DETANONOate, a nitric oxide generator, scavenged superoxide to 31%. Both TTM and DETANONOate decreased the number of superoxide positive cells to that of non-transgenic astrocytes. Standard error bars are not displayed because these values represent the absolute number of cells counted out of 412 total cells. In each of three different astrocyte prepartions, astrocytes were first assessed for superoxide generation, and then treated with inhibitors.

Figure 6.3 SOD1<sup>G93A</sup> displayed a temporal accumulation with the mitochondria. In younger cultures, some SOD1<sup>G93A</sup> transgenic astrocytes displayed a diffuse, cytosolic fluorescence for SOD1 (A). In cultures analyzed at a later time point SOD1<sup>G93A</sup> astrocytes displayed a more punctate, mitochondrial-like fluorescence (B; see also Fig. 6.1). Interestingly, astrocytes with cytosolic expression of SOD1<sup>G93A</sup> did not show increased superoxide generation and HO-Mito-Et<sup>+</sup> fluorescence (1-10), as compared with the increased superoxide generation and HO-Mito-Et<sup>+</sup> fluorescence observed with mitochondrial localized SOD1<sup>G93A</sup> (11-20).

Figure 6.4 SOD1<sup>G93A</sup> astrocytes have increased superoxide scavenging activity as compared to non-transgenic astrocytes, as measured by the NBT activity gel. Protein concentrations were determined from lysed astrocytes. These two bands are from the same gel, although excised to place samples of equal protein concentration adjacent for comparison and clarity.

6.7 Figures

Figure 6.1





Figure 6.2

| SOD <sup>G93A</sup> |    | HO-Mito-Et <sup>+</sup> |            |                |       |
|---------------------|----|-------------------------|------------|----------------|-------|
|                     |    | Exc=514 nm              | Exc=405 nm | Merge          | MERGE |
|                     |    | 2                       | 3          | 4              | 5     |
|                     | 6  | 7 ~ .                   | 8          | 9              | 10    |
| (B)                 | 11 | 12                      | 13         | -14            | 15    |
|                     | 16 | 17                      | 18         | 19<br>19<br>19 | 20    |



Figure 6.4

Chapter 7

**Concluding Remarks** 

In the 14 years that have lapsed since the discovery that mutations to SOD are linked to ALS, the question of whether free radicals are involved in the disease process – epitomized by the question "Did Free Radicals Kill Lou Gehrig?" - remains an unresolved issue. The reduction of molecular oxygen by one election to form superoxide is a pivotal step in producing free radicals. implications The range of for superoxide generation includes neurodegenerative disorders (36-40), aging (36-38), cancer (37) and diabetes (41). However, the lack of a sensitive and selective means to detect superoxide in different compartments of the cell has greatly hampered the progress of determining the role of superoxide in these diseases (52). We developed a method that can detect superoxide with increased selectivity by detecting the superoxide specific, hydroxylated product of hydroethidine (HE) and MitoHE (Chapters 4 & 5).

We propose the use of HE and Mito-HE is also advantageous because its mechanism of oxidation suggests HE and Mito-HE will effectively compete with SOD for superoxide. The second step of the reaction between HE with superoxide was proposed to involve radical-radical coupling between the HE radical cation and superoxide anion (Chapter 4). This reaction will occur at a rate limited most likely by diffusion, making HE an efficient competitor with endogenous SODs for superoxide.

HE and Mito-HE differ in the location of intracellular accumulation. HE will readily diffuse through cell membranes and can be oxidized inside a cell. HE oxidation products, ethidium or hydroxy-ethidium, intercalate into DNA to emit red fluorescence from within the nucleus, or to a lesser extent, diffusely throughout the cytosol (Figure 4, Introduction). Therefore, the site of superoxide generation will generally be unknown. On the other hand, Mito-HE preferentially accumulates into the mitochondria. Therefore Mito-HE can be utilized to answer a more specific question of whether superoxide production is being increased within the mitochondria. We have thoroughly characterized methods to use Mito-HE to detect mitochondrially generated superoxide in

isolated mitochondria and live cells (Chapter 5). The next step will be to determine where in the mitochondria superoxide is being generated. A method which pinpointed the site of oxidant production within the mitochondria, whether it be in the inner membrane space, the matrix, the inner or outer membranes or a particular complex within the inner membrane, would provide information on a direct target to inhibit superoxide generation.

The use of Mito-HE to detect mitochondrial superoxide provided strong evidence that the over-expression of the ALS associated mutation, SOD<sup>G93A</sup>, increases superoxide generation in astrocytes (Chapter 6). Astrocytes are key components of the central nervous system involved in the intricate interplay between motor neurons, interneurons, oligodendrocytes and microglia (73). Astrocytes are crucial for disease propagation in ALS (75, 76). Selectively knocking down mutant SOD1 in glia slows disease progression (76). In vitro assays have shown that astrocytes expressing mutant SOD<sup>G93A</sup> do not die themselves, but can induce apoptosis in non-transgenic motor neurons (31). However, how the SOD<sup>G93A</sup> mutation confers this toxic gain of function is not well understood.

We were able to determine that SOD<sup>G93A</sup> expression causes mitochondria in astrocytes to become dysfunctional and generate more superoxide (Chapter 6). The difference between SOD<sup>G93A</sup> and non-transgenic astrocytes was clearly observable because transgenic astrocytes and nontransgenic astrocytes were cultured together. This allowed for a direct comparison of superoxide generation in SOD<sup>G93A</sup> astrocytes adjacent to nontransgenic astrocytes. This method is advantageous because it eliminates observable differences in fluorescence that can arise in cultures analyzed separately, due to instrument settings or culture conditions. Superoxide detection was confirmed using two excitation wavelengths. As an additional control, superoxide generation was inhibited using nitric oxide, which reacts at diffusion limited rates and effectively competes for superoxide. The decreased membrane potential of the mitochondria in SOD<sup>G93A</sup> astrocytes may compromise and reduce the accumulation of Mito-HE. Therefore, we may have underestimated the actual increase in superoxide generation, which may have been exacerbated if membrane potentials were equivalent.

The increase in superoxide generation in SOD<sup>G93A</sup> astrocytes was inhibited by the copper chelator, tetrathiomolybdate (TTM), implying superoxide generation was copper-dependent. SOD is present within the inner membrane space of the mitochondria (142). The high demand caused by grossly over-expressed SOD could create a state where many SODs within the innermembrane space are partially metallated. Partially metallated SODs containing copper and not zinc could be catalyzing the formation of oxidants, such as superoxide. Superoxide generation can in turn damage respiratory chain complexes compounding superoxide generation from the respiratory chain as well.

The increase in superoxide generation correlated with intense fluorescence from SOD<sup>G93A</sup> labeling, indicating both superoxide generation and SOD<sup>G93A</sup> expression was localized at the mitochondria. This finding supports previous reports of mutant SOD associating with the mitochondria (77). Considerable evidence points to mitochondria becoming dysfunctional in ALS and the mitochondria have been proposed as a key target for triggering motor neuron death in ALS (131). Transgenic SOD<sup>G93A</sup> mice and ALS patients show peculiar mitochondrial morphologies, such as giant vacuolization and aggregation (125). Moreover, mitochondria abnormalities within SOD<sup>G93A</sup> transgenic mice are observable prior to visible signs of muscle weakness, and therefore mitochondria have been proposed to play a causal role in ALS pathogensis (129).

This work supports that the expression of the ALS-linked SOD<sup>G93A</sup> mutation causes mitochondrial dysfunction. Increased free radical production from the mitochondria can damage respiratory complexes, compromising oxidative phosphorylation. Increased free radicals may also damage mitochondrial DNA causing organelle dysfunction. Mitochondria are key

organelles capable of determining the fate of a cell (143). Loss of mitochondrial function due to mitochondrial DNA mutations causes severe phenotypes such as deafness, diabetes and blindness (46). Damaged mitochondria can trigger cell death through the release of pro-apoptotic proteins (143).

One question in the study of ALS is why is there a selective loss of motor neurons? Motor neurons can extend extremely long axons measuring up to a meter in humans. Thus mitochondria migrating along axons can be far removed from the nuclear encoded proteins that account for 99% of the proteins in mitochondria. The large size of motor neurons and metabolically active neuromuscular junctions also confer an extremely high metabolic demand from the mitochondria. The especially high demand of motor neurons upon their mitochondria may explain why motor neurons are vulnerable in ALS. Indeed, ALS transgenic mice selectively lose larger motor neurons (initially those > 25  $\mu$ m) in the ventral horn (144).

The detection of superoxide may be important to understand how to counter the molecular mechanisms driving the progressive death of motor neurons in ALS. Curiously, superoxide itself is not the "super" oxidizing species its name implies, but is a mild reductant at neutral pH. A major mechanism that increases the toxicity of superoxide is most likely facilitated through its reaction with nitric oxide to generate peroxynitrite. Peroxynitrite is a strong oxidant capable of damaging proteins, nucleic acids, carbohydrates and lipids (7). Chapter 1 described a method for synthesizing peroxynitrite, but also reviewed important factors for the study of peroxynitrite, to increase the validity of experiments determining the role of peroxynitrite in disease.

Peroxynitrite facilitates oxidative damage through two distinct pathways mediated by either peroxynitrite anion or peroxynitrous acid (Figure 1, Chapter 1) (7). Peroxynitrite anion can directly oxidize metal centers (7). On the other hand, peroxynitrite can be protonated to generate peroxynitrous acid. Peroxynitrous acid is highly unstable and rapidly decomposes. Approximately 30% of peroxynitrous acid decomposes into a highly oxidizing intermediate, presumably hydroxyl and nitrogen dioxide radicals, which can nitrate proteins and lipids. The anti-oxidant urate is capable of distinguishing between these two types of oxidation processes. Urate does not scavenge peroxynitrite anion directly, but inhibits peroxynitrous acid-mediated nitration of proteins (19). One report proposed that urate itself was nitrosated by peroxynitrite (97). However in Chapter 3 we used mass spectrometry to determine the structure of peroxynitrite-oxidized urate, and proposed the novel product, triuret. Fragmentation of triuret produced a mass spectrum identical to that observed for peroxynitrite-oxidized urate.

Because of the manner in which urate reacts with peroxynitrite decomposition products and not peroxynitrite anion itself, urate can be used to parse the mechanism of peroxynitrite-mediated damage. We collaborated in a project lead by Dr. Alvero Estevez which investigated how peroxynitrite induces apoptosis in neuronal cells (PC-12 cells). Previously, it was not clear whether peroxynitrite induced apoptosis as a direct or indirect oxidant. Dr. Estevez found tyrosine containing peptides blocked peroxynitrite-mediated apoptosis (145). Peptides containing other amino acids, such as cysteine or methionine, did not block apoptosis (145). Similar to urate, tyrosine containing peptides do not directly consume peroxynitrite anion, as measured by stopped flow spectroscopy (Figure 7.1) (145). These results imply it is the radical



intermediates of peroxynitrous acid which are involved in peroxynitritemediated apoptosis of neuronal cells.

One exciting new target of peroxynitrite-derived radicals that we uncovered is nerve growth factor (NGF). A collaborative project lead by Drs. Pehar and Barbeito (Pasteur Institute of Uruguay) revealed treatment with peroxynitrite transforms NGF into an apoptotic inducing factor, increasing the potency of NGF to induce apoptosis 10,000 fold (Figure 7.2) (146). Thus, peroxynitrite may not only mediate its toxic actions through the oxidation of proteins and the subsequent loss of protein function, but also through oxidative action which confers a toxic gain of protein function.

Nerve growth factor (NGF) is a trophic factor involved in neuronal differentiation and survival, however NGF can also induce apoptosis through the p75 neurotrophin receptor ( $p75^{NTR}$ ) (29).  $p75^{NTR}$  is not expressed in



Figure 7.2. Ribbon diagram of NGF showing residues involved in peroxynitrite oxidation, as determined by mass spectrometry.

typical adult motor neurons however can be re-expressed following nerve injury, axotomy (147-149) and in neurodegenerative disorders such as ALS Therefore we investigated the mechanism of NGF-induced (150, 151). apoptosis via activation of p75<sup>NTR</sup> on motor neurons. We utilized the selective method of superoxide detection we developed, and found apoptosis induced by NGF involves increased mitochondrial superoxide generation (Pehar, et. al; manuscript submitted J Neurosci). Moreover, superoxide generation and inhibited apoptosis neutral sphingomyelinase was by inhibitors. Sphingomyelinases generate ceramides and ceramide generation has been proposed to be a mediator of p75<sup>NTR</sup>-induced apoptosis (152, 153). These results suggest NGF induces apoptosis through the p75<sup>NTR</sup> receptor by activating sphingomyelinases and generating ceramides. Ceramides can damage mitochondria (154) and accordingly we observed mitochondrial dysfunction and increased superoxide generation. Raising antioxidant defenses by Nrf2 activation can prevent NGF-induced apoptosis. Therefore increasing antioxidants through the pharmacological activation of Nrf2 may prevent apoptosis in pathologies associated with the re-expression of p75<sup>NTR</sup>, such as ALS.

The role of oxidants in ALS remains controversial and the exact mechanism of how SOD mutations lead to motor neuron disease is unknown. It is clear that mutant SODs obtain a toxic gain of function, as opposed to a loss of SOD function to dismutate superoxide. Familial ALS involving SOD mutations occurs independently of and without correlation to the activity of the SOD mutant (2). The different level of activity displayed by the different ALS-associated SOD mutants does not correlate with disease severity (155, 156). For example, expression of SOD<sup>G85R</sup>, a putatively inactive mutant, still leads to motor neuron disease (157). In addition, SOD null mice do not develop ALS (158), supporting the fact that a loss of SOD activity does not cause motor neuron disease.

A common feature observed between the mice expressing mutant SOD is the accumulation of unbiquitin positive, SOD aggregates in motor neurons and astrocytes (2). Protein aggregation is a feature commonly observed in neurodegenerative diseases, such as the accumulation of tau and amyloid in Alzheimer's disease (159),  $\alpha$ -synuclein in Parkinson's disease (159) and huntington in Huntington's disease (160). The role of aggregates in neurodegeneration is unclear. Are aggregates toxic species causing cell death; are they just a tombstone marking death; or could aggregates be protective by sequestering toxic species?

The theory that aggregation is the toxic element causing motor neuron disease is problematic because it does not explain the other 98% of ALS cases arising sporadically. Alternatively, sporadic and familial cases could be explained through the proposed toxic actions of zinc deficient SOD (161). One common phenotype between some ALS mutations is a decreased affinity for zinc (162). The loss of zinc from SOD transforms SOD into a protein capable of inducing motor neuron death (133). Both wild type and mutant SOD are toxic once they become zinc deficient (133). This theory may explain the other 98% of sporadic ALS cases, where other conditions besides ALS mutations have caused SOD to become zinc deficient, thereby initiating motor neuron death.

The toxicity of zinc deficient SOD has been proposed to be mediated through the aberrant redox chemistry of its copper atom (133). Copper replete, zinc deficient SOD catalyzes the formation of peroxynitrite in the presence of nitric oxide (133). This work found that the copper chelator, TTM, inhibited superoxide generation associated with mutant SOD expression in astrocytes. Increased oxidant production and damaged mitochondria play a crucial role on cell survival and may be critical in ALS pathogenesis.

Therefore, antioxidant agents based upon superoxide scavenging or mitochondrial dysfunction should be efficacious in the treatment of ALS. Indeed, antioxidants, such as epigallocatechin (163), melatonin (164), iron

porphyrin (FeTCCP) (165), SOD/catalase mimetics and pharmacological catalytic antioxidants (166) extend survival in ALS transgenic mice. Administration of the antioxidant, edaravone, decreased the rate of decline in ALS patients and decreased the level of nitrotyrosine in cerebral spinal fluid (167). The mitochondrial targeted antioxidant, Mito-TEMPOL, has been shown to be efficacious in extending the life-span of SOD<sup>G93A</sup> transgenic mice (168). The superoxide scavenger MnTBAP was also efficacious at inhibiting disease in SOD<sup>G93A</sup> transgenic mice, and a similar compound, AEOL, is currently in phase II trials for the clinical treatment of ALS patients.

The overall goal of this work is to help determine under what disease states oxidants such as superoxide are generated and to better understand the mechanism of oxidants such as peroxynitrite. The final application is to use this knowledge to combat neurodegeneration in hopes to better develop therapies to tragic diseases, such as Lou Gehrig's disease.

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## APPENDIX

## Appendix 1. The administration of urate to mice expressing the ALSlinked SOD<sup>G93A</sup> mutation

Because of urate's anti-oxidant character and ability to inhibit peroxynitritemediated nitration of tyrosine residues, urate was administered to mice overexpressing the ALS-associated SOD<sup>G93A</sup> mutation. Mice were orally administered a diet supplemented with 3% uric acid (UA) and 2% oxonic acid (OA) beginning at eight weeks of age. Oxonic acid is an inhibitor of the enzyme, uricase, which degrades uric acid in rodents. Fifty four female mice were split pairwise so that one littermate was supplemented with the UA/OA diet and the other received the control diet. Similarly, twenty eight male mice were also split pairwise having one littermate receiving UA/OA. Administration of the diet did not alter ALS mice feeding habits or weight gain and raised plasma uric acid levels approximately two fold (Figure A1). Supplementation with UA/OA failed to affect onset of disease, as assessed by the paw grip endurance test, in either female (Figure A2) or male (Figure A3) mice. In addition, the UA/OA diet failed to increase lifespan in either female (Figure A4) or male (Figure A5) mice.

In a mouse model of multiple sclerosis, intraperitoneal injections of urate administered four times daily inhibited paralysis, tissue damage, and tyrosine nitration (26). Similar administration of higher doses, in contrast to those obtained by oral administration, may be required to inhibit tyrosine nitration and paralysis in SOD<sup>G93A</sup> ALS mice.



Figure A1. HPLC separation of plasma to detect uric acid, eluting at 3.1 min ( $\lambda$ =292 nm). The pink trace is representative of plasma from mice receiving control diet and the blue trace from UA/OA supplemented mice.



Figure A2. Assessment of paw grip endurance to determine the age of disease onset in female, SOD<sup>G93A</sup> mice. Paw grip endurance is tested by placing a mouse on a wire screen and then inverting the screen and recording the length of time mice hold on to the screen. Mice are tested for 90 seconds. Initially, all mice can hang for 90 second, however their ability to hold declines as the disease progresses.



Figure A3. Assessment of paw grip endurance to determine the age of disease onset in male, SOD<sup>G93A</sup> mice.



Figure A4. Survival plot of the lifespan of female, SOD<sup>G93A</sup> mice.



Figure A5. Survival plot of the lifespan of male, SOD<sup>G93A</sup> mice.