EFFECTS OF TAURINE AND HYPOTAURINE
ON OXIDATIVE LUNG INJURY

by

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ABSTRACT

The present studies are based on the premise that pulmonary injury, during periods of hypoxia, ischemia, and reperfusion, may be due to increased production of reactive oxygen species, including the superoxide anion (O$_2^-$), hydroxyl radical (•OH), and hydrogen peroxide (H$_2$O$_2$), and on the premise that this injury can be ameliorated by antioxidant pre-treatment.

The sulfur-containing β-amino acids, taurine and its precursor, hypotaurine, have been shown indirectly to possess antioxidant properties by several investigators. The mechanism(s) by which taurine and hypotaurine exert their antioxidant effects has(have) remained unclear despite many years of intensive study, as does the precise physiological role for these two β-amino acids.

The goals of the present study were: 1) to evaluate the effects of taurine and hypotaurine in experiments that model biochemical events which are believed to be important components of oxidative pulmonary injury; 2) to assess the potential antioxidant ability of the amino acids by determining their capacity to scavenge the free radicals, •OH and O$_2^-$, directly; and 3) to investigate the effect of these amino acids on reperfusion injury of rat lungs in an ex vivo ischemia-reperfusion injury model.

The results of this study indicate that taurine and hypotaurine are not effective in detoxifying H$_2$O$_2$ and, in fact, taurine was found to augment H$_2$O$_2$ production in phorbol myristate acetate-stimulated macrophages. At 26, 78, and 104 mM, taurine was found to elevate H$_2$O$_2$ production 13%, 28%, and 43%, respectively, above the
positive control. Taurine (5-120 mM) and hypotaurine (2-10 mM) were also ineffective (p > 0.05) in protecting biomembranes against free radical-induced lipid peroxidation. However, taurine (10-300 mM) and hypotaurine (2-30 mM) were found to possess the ability to scavenge hydroxyl radicals. Taurine (148 and 193 mM) and hypotaurine (19 mM) were found to possess the ability to scavenge superoxide at the high end of the concentration range tested. This was demonstrated by the ability of these amino acids to compete with both ferricytochrome c for available O$_2^{-}$ and deoxyribose for available ·OH, within the respective systems designed to produce these two reactive species. Additionally, in an EPR study using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap, both taurine and hypotaurine caused dose-dependent inhibition of DMPO-OH and DMPO-OOH adduct formation. In the ex vivo rat lung model, the addition of 5 and 10 mM taurine to the perfusion medium 20 minutes prior to the induction of ischemia appeared not to provide significant protection (p > 0.05) against reperfusion injury to isolated rat lungs exposed to 60 minutes of ischemia followed by 30 minutes of reperfusion. However, the data obtained from the ex vivo lung experiments was variable and must be interpreted with caution. Furthermore, in preliminary studies it was found that 50 mM taurine may be toxic to the isolated, perfused, rat lung.

In conclusion, the antioxidant properties of taurine and hypotaurine are due to their capability to scavenge some of the reactive species of oxygen. The apparent inability of low concentrations of taurine to ameliorate post-ischemic reperfusion injury of lungs is consistent with the fact that relatively high concentrations of taurine were needed for the amino acid to demonstrate significant scavenging of O$_2^{-}$ and ·OH.
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LIST OF ABBREVIATIONS

O$_2^-$ Superoxide anion radical
·OH Hydroxyl radical
OH$^+$ Hydroxyl ion
H$_2$O$_2$ Hydrogen peroxide
O$_2^-$ Singlet oxygen
EPR Electron paramagnetic resonance
DMPO 5,5-dimethyl-1-pyrroline-N-oxide
DMPO-OH Spin adduct of the hydroxyl radical and DMPO
DMPO-OOH Spin adduct of the superoxide radical and DMPO
π* 2p Antibonding 2p molecular orbital
e$^-$ electron
H$^+$ Hydrogen ion
SOD Superoxide dismutase
DNA Deoxyribonucleic acid
UV Ultraviolet
GSH Reduced glutathione
M$n^+$ Oxidized form of transition metal
M$^{n+}$ Reduced form of transition metal
LOO$^.$ Lipid peroxide
LOOH Lipid hydroperoxide
LO$^.$, L$^.$ Lipid fragmentation products
Fe$^{2+}$ Ferrous iron
Fe$^{3+}$ Ferric iron
O$_2$ Molecular oxygen
HOCI Hypochlorous acid
Cl$^-$ Chloride ion
DCF 2', 7' -dichlorofluorescein, fluorescent compound
DCFH-DA 2', 7' -dichlorofluorescin-diacetate, non-fluorescent analog
DCFH 2', 7'-dichlorofluorescin, non-fluorescent analog
HBSS Hank's Balanced Salt Solution
PMA Phorbol myristate acetate
DMEM Dulbecco's Modified Eagle Medium
EDTA Ethylenediamine tetraacetic acid
FeCl$_3$ Ferric chloride
ADP Adenosine diphosphate
NADPH Nicotinamide-adenine dinucleotide phosphate (reduced)
TBA Thiobarbituric acid
MDA Malondialdehyde
MnCl$_2$ Manganese chloride
DETAPAC Diethylenetriamine pentaacetic acid
<table>
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<tr>
<td>KH₂PO₄-KOH</td>
<td>Potassium phosphate, potassium hydroxide buffer</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>Pₐ</td>
<td>Pulmonary artery pressure</td>
</tr>
<tr>
<td>Pₐₘₚ</td>
<td>Peak airway pressure</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>LSD</td>
<td>Least significant difference</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid-reactive substances</td>
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<tr>
<td>AAPH</td>
<td>2,2'-Azobis(2-amidinopropane)dihydrochloride</td>
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Chapter 1

REVIEW OF THE RELATED LITERATURE

A. Free Radicals

Free radicals are short-lived, highly reactive molecules that may be organic or inorganic in nature. These molecules are capable of initiating chain reactions and have an unpaired electron in their outer shell. Free radicals may be of three chemically distinct forms: a cation radical, which is produced during one-electron oxidation by removal of a π-electron; an anion radical, which is generated by one-electron reduction by addition of an electron to the π orbital; and a carbon-centered radical, which is usually produced by homolytic cleavage of a C-H bond (1). The reactivity of free radicals varies depending on both their nature and several characteristics of their environment, including temperature, pH, and molecular concentration. The potentially damaging effects of free radicals in living systems have been well established. In fact, free radicals and antioxidant therapy have become very popular subjects outside the realm of the scientific community. Public awareness of free radicals is a result of increasing publicity and advertising which implicate the involvement of free radicals in many biological processes, including aging, cancer, and environmental toxicant exposure (2).

Molecular oxygen possesses an electron configuration that makes it capable of forming reactive oxygen species as intermediates during its reduction to form water. The orbitals in the outer shell of atomic oxygen do not contain the allowed full complement of two electrons each. When two atoms of oxygen form molecular oxygen, the electrons in the orbitals of the outer shell of one oxygen atom are shared with those in the outer orbital of a second oxygen atom, forming a new molecular orbital and a
covalent bond. With molecular orbitals, Hund's Rule and the Pauli Exclusion Principle must be satisfied, i.e., only two electrons may occupy the same orbital, and then only when they have opposite spins. Although molecular oxygen has an even number of electrons, the two electrons in molecular oxygen that are to occupy the outer orbitals are not of opposite spin, so each one of them resides, unpaired, in a separate π*2p orbital (Scheme A) making ground state oxygen a "diradical".

<table>
<thead>
<tr>
<th>( \sigma^* 2p )</th>
<th>( \pi^* 2p )</th>
<th>( \pi 2p )</th>
<th>( \sigma 2p )</th>
<th>( \sigma^* 2s )</th>
<th>( \sigma 2s )</th>
<th>( \sigma^* 1s )</th>
<th>( \sigma 1s )</th>
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<tr>
<td>( \uparrow \downarrow )</td>
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Ground-state \( O_2 \)  \( \Sigma\)-Singlet \( O_2 \)  Superoxide  Peroxide ion  \( \Delta\)-Singlet \( O_2 \)

**Scheme A: Electron Arrangement of Oxygen Species**
Metabolic redox reactions normally involve the exchange of two electrons in a spin-paired arrangement. For oxygen to accept two such electrons at the same time would require that one of its outer electrons undergo a thermodynamically unfavorable spin inversion. The diradical nature of ground state oxygen circumvents the problem of spin inversion by making oxygen capable of multiple, spontaneous, univalent reductions, with the consequence being the formation of partially-reduced oxygen intermediates. These intermediates are either free radicals themselves or highly reactive species capable of initiating or participating in reactions which may lead to the formation of free radicals. Free radicals are potent oxidizing or reducing agents and, as such, represent a severe threat to biological building blocks, such as proteins, lipids, and carbohydrates, which are susceptible to oxidation and reduction.

Oxygen centered free radicals are of particular biological importance. In living systems, which use the reduction of oxygen to water and the simultaneous oxidation of organic nutrients to carbon dioxide as a means to produce energy for driving metabolic reactions, the production of reactive species of oxygen is a natural consequence (3). Under normal circumstances, the majority of molecular oxygen (95-98%) undergoes a concerted tetravalent enzymatic reduction to two molecules of water by the mitochondrial cytochrome oxidase system, with no detectable release of toxic, partially reduced, oxygen intermediates. However, 1-2% of the molecular oxygen delivered to the mitochondria "leaks" from the tetravalent pathway and undergoes univalent reduction (4). This alternate pathway of oxygen reduction does produce several highly reactive species including H$_2$O$_2$, •OH, and possibly O$_2^·$. Oxygen can also accept a total of four electrons to form two water molecules via a series of univalent, non-enzymatic reactions (Scheme B) that also produce the intermediate products superoxide
anion radical, hydrogen peroxide, and the hydroxyl radical (5). Each of these species poses a significant threat to cellular integrity because of the potential that exists for their interaction with and degradation of biologically vital molecules, such as DNA, proteins and membranes, by means of oxidation and reduction.

\[
\begin{align*}
\text{O}_2 + e^- & \rightarrow \text{O}_2^- \\
\text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + e^- & \rightarrow \text{OH}^- + \text{H}_2\text{O} \\
\text{OH}^- + \text{H}^+ & \rightarrow \text{H}_2\text{O}
\end{align*}
\]

**Scheme B: Univalent Reduction of a Series of Various Oxygen Species**
A means of intracellular detoxification of potentially damaging species of oxygen produced during oxidative metabolism does exist. Enzymatic defenses include the superoxide dismutases (SODs), catalases, and peroxidases (Scheme C). SOD is present in most tissues and catalyzes the simultaneous oxidation and reduction of two superoxide anion radicals to form molecular oxygen and hydrogen peroxide, respectively. Catalases and peroxidases then catalyze the reduction of the hydrogen peroxide to water, a completely innocuous end product. Additionally, there are small molecular weight antioxidant molecules, such as ascorbic acid, \( \alpha \)-tocopherol, and glutathione, present in the intra- and extracellular environment which are able to stop the free radical-initiated chain reaction events. This system of defenses plays a crucial role in protecting the cell from injury. For example, failure of this system to inactivate reactive oxygen species has serious consequences, including degradation of hyaluronic acid and collagen, thereby rendering serious damage to the connective tissues (6), degradation of polyunsaturated fatty acids leading to membrane damage (6), interaction with nucleic acids in a way that damages DNA (7), and interaction with proteins such as enzymes or structural proteins rendering them inactive or structurally ineffective, respectively (8).

One of the important types of cellular damage caused by free radicals is damage to the cellular and subcellular membranes. This type of damage is initiated by superoxide anions and hydroxyl radicals oxidizing structurally important polyunsaturated membrane phospholipids. The process may be propagated by secondarily-derived lipid peroxide radicals, lipid hydroperoxides, and lipid fragmentation products that are also oxidants (Scheme D). This process is termed "lipid peroxidation," and transition
metals, such as iron, are believed to play an important role in its initiation and propagation (9).

In response to single electrons generated by the action of UV Light, radiation, phagocytosis, autoxidation, oxido-reductases, and respiratory chain enzymes, molecular oxygen may become reduced:

\[
\text{O}_2 + e^- \rightarrow \text{O}_2^-. 
\]

The cell's natural defense against the production of superoxide radical is the enzyme superoxide dismutase:

\[
\text{O}_2^- + \text{O}_2^- \xrightarrow{2\text{H}^+} \text{H}_2\text{O}_2 \\
\text{Superoxide Dismutase (SOD)}
\]

The hydrogen peroxide produced by the dismutation of superoxide is removed by either catalase or glutathione peroxidase enzymes:

\[
\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} \\
\text{Catalase and / or Glutathione Peroxidase}
\]

If hydrogen peroxide does not get reduced to water by catalase or glutathione peroxidase, it may react with available iron to produce the hydroxyl radical:

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

Hydroxyl radicals are very reactive and will interact readily with many biologically important substances. The small molecular weight antioxidant molecules such as Glutathione, Vitamin C, and Vitamin E are capable of stopping the chain reactions initiated by the hydroxyl radical that would otherwise lead to DNA damage, lipid peroxidation, and protein damage:

\[
\cdot\text{OH} + e^- \xrightarrow{\text{H}^+} \text{H}_2\text{O}
\]

Scheme C: Antioxidant Defenses of the Cell
Scheme D: Initiation and Propagation of Free Radical-Induced Lipid Peroxidation

\[ \text{Initiation: } \cdot \text{OH}, \text{O}_2^1, \text{O}_2^{-}, \quad \text{LH} \rightarrow \text{L}^\cdot + \text{H}_2\text{O} \]

\[ \text{Propagation: } \text{LOO}^\cdot + \text{LH} \rightarrow \text{LOOH} \]

\[ \text{LOOH} + \text{Me}^{n+1} \rightarrow \text{LO}^\cdot + \text{Me}^{n+1} \]

\[ \text{LOOH} + \text{Me}^{n+} \rightarrow \text{LO}^\cdot + \text{Me}^{n+1} \]

\[ \text{O}_2^1 = \text{singlet oxygen} \]

\[ \text{LH} = \text{lipid oxidation substrate} \]

\[ \text{Me}^{n+1} \text{ and } \text{Me}^{n+} = \text{oxidized and reduced forms of transition metals} \]

\[ \text{LOO}^\cdot = \text{lipid peroxide, LOOH = lipid hydroperoxide,} \]

\[ \text{LO}^\cdot, \text{L}^\cdot = \text{lipid fragmentation products} \]

The chain reaction of cellular destruction will continue until it is terminated by interaction of the reactive species with antioxidant molecules. If the process continues long enough, it will affect the cellular envelope. It is possible that the membranes of organelles, such as the mitochondria and lysosome, then may be disrupted by similar means. Lysosomal membrane degradation leads to further destruction of the cell by virtue of release of digestive enzymes which normally assist in lysis carried out within the lysosome. Damage to the mitochondria would expose the cell to yet another source of superoxide anion radical produced by the "leaking" of molecular oxygen from the mitochondrial electron transport system (4).

The involvement of free radical injury in a wide range of disease processes is not surprising in light of the wide-spread occurrence of systems that produce reactive species of oxygen in vivo and the extent of damage they may cause to universally important cellular and extracellular components. A review of a long list of clinically important situations in which free radicals are believed to play a role in tissue injury is presented by Bulkley (2). For the purpose of this study, the focus will be directed toward ischemia/reperfusion syndromes, i.e., diseases or surgical procedures which involve a period of making the tissue ischemic followed by reperfusion of the same tissue. Examples of clinical situations which involve ischemia and reperfusion include myocardial infarction, cardiopulmonary bypass, organ preservation and transplantation, cerebral ischemia, limb ischemia, and free skin flap transfers.
B. Ischemia-Reperfusion Injury

The term ischemia is used to describe a period of hypoxia due to occlusion of blood supply to a tissue. Ischemia may occur in a living system as a result of a pathological process or it may be induced as part of a surgical procedure to correct some other problems. The process termed reperfusion refers to the post-ischemic period whereby removal of the occlusion re-establishes blood flow and the tissue becomes re-oxygenated.

Recent studies have provided evidence that extensive tissue damage occurs in the post-ischemic tissue during re-oxygenation following a period of ischemia (10-13). Oxidative processes are believed to be involved in ischemia-reperfusion injury because lipid peroxidation (14) and free radical production (15, 16), as well as oxidation of soluble proteins (17), have been measured during this type of injury, indirectly implicating reactive species of oxygen as causative agents. The production of reactive oxygen species during the ischemic period has been demonstrated in several tissues, including heart, intestine, and lung (18, 19, 20). The reactive oxygen species include the superoxide anion radical, the hydroxyl radical, and hydrogen peroxide. Various sources have been proposed for the intracellular production of these oxidants, including the mitochondrial electron transport chain (21), the metabolism of arachidonic acid by lipoxygenase and cyclooxygenase (22), and the reaction mediated by xanthine oxidase which converts hypoxanthine to xanthine and uric acid (23). The role in ischemia reperfusion injury of phagocytic cells, such as neutrophils and macrophages, which utilize reactive oxygen species to kill engulfed bacteria, is not clear. However, it has been suggested by some that these cell types as well as other blood components, such as
platelets, may play an important role in the induction and/or propagation of oxidative injury (24-26).

Although ischemia reperfusion injury has been partially characterized in the lung, the exact biochemical and physiological mechanisms involved still remain obscure (24, 27-31). In general, the mechanism of post-ischemic reperfusion injury in other tissues, such as heart, brain, intestine, liver, pancreas, kidney, and skin, are very similar to that of the lung. However, there are obvious physiological and anatomical differences between these tissues and the lung which make the lung a unique organ to study for this type of injury. The lung affords the unique opportunity of allowing the physiological separation of perfusion and hypoxia due to its unique blood supply and anatomical arrangement designed for gas exchange. For example, if ventilation of the lung remains constant, it is possible to have a situation whereby the induction of lung ischemia does not render the organ hypoxic. In fact, it can be argued that this situation would elevate the alveolar partial pressure of oxygen, thereby increasing oxygenation of the lung tissue. Fisher et al. found evidence that elevated partial pressures of oxygen during lung ischemia accelerate the rate of tissue injury (20). This particular property of the lung may explain, at least in part, why reperfusion after ischemic insult may not be necessary for oxidative damage to occur in the lung. Thus, under certain circumstances where ischemia may exist but ventilation remains constant, the lung may be more susceptible to oxidative damage than are other tissues.

Another important characteristic of the lung is that it is well endowed with cytochrome P-450 enzymes (32). These microsomal enzymes have been shown to contribute a majority of the superoxide anion radical and hydrogen peroxide generated by lung
under normal conditions (33) as well as during hyperoxia (34-35). Bysani et al. propose that the cytochrome P-450 enzyme system may play an additional role in reperfusion injury by providing an intracellular source of iron (36). Iron may promote tissue injury by catalyzing the production of the hydroxyl radical via the Fenton reaction (Scheme E) and by propagating lipid peroxidation (Scheme D). It is now generally believed that the Haber-Weiss reaction (Scheme E) is also catalyzed by iron in the following reaction:

\[
\begin{align*}
O_2^{-\cdot} + Fe^{3+} & \rightarrow Fe^{2+} + O_2 \\
H_2O_2 + Fe^{2+} & \rightarrow \cdotOH + OH^- + Fe^{3+}
\end{align*}
\]

Overall reaction:

\[
H_2O_2 + O_2^{-\cdot} \rightarrow \cdotOH + OH^- + O_2
\]

Iron chelation by deferroxamine has proven effective in reducing reperfusion injury in the lung (31), as well as in various other tissues, indicating an important role for iron in this type of injury. Bysani et al. conclude that the cytochrome P-450 system serves as an important source of iron in mediating oxidant injury to the rabbit lung during reperfusion (36).

Specific situations in which lung ischemia-reperfusion injury is of relevance are cardiopulmonary bypass, pulmonary arterial embolectomy, acute respiratory distress syndrome, and heart and/or lung transplantation techniques. This type of oxidative injury in the lung also has been documented by Jackson et al. in re-expansion of atelectatic lung (37).
Fenton Reaction

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

Haber-Weiss Reaction

\[ \text{H}_2\text{O}_2 + \text{O}_2^- \cdot \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_2 \]

Scheme E: Free Radical-Producing Biological Reactions
C. Detection of Reactive Oxygen Species

H₂O₂ Production by Macrophages

The respiratory burst of phagocytic cells can be measured by monitoring the intracellular oxidation of 2',7'-dichlorofluorescin (38,39). The principle of this method is based on evidence which strongly suggests that the non-fluorescent compound, 2',7'-dichlorofluorescin-diacetate (DCFH-DA), is membrane permeable and will be taken in by viable cells and enzymatically cleaved by esterases in the cytosol to form the non-fluorescent analog, 2',7'-dichlorofluorescin (DCFH). Upon removal of the acetate groups, the DCFH becomes trapped intracellularly. When the respiratory burst is activated, the DCFH is converted to the fluorescent compound 2',7'-dichlorofluorescein (DCF) via the action of H₂O₂ and peroxidase enzymes. The fluorescent compound is localized mainly within peroxidase-containing granules thereby making it a primary measure of the production of reactive oxygen intermediates within the phagosome (40). The production of H₂O₂ may be measured using flow cytometry or microfluorimetry techniques. In previous studies, this method of H₂O₂ detection has been useful in determining the ability of potential antioxidant substances to interact with this particular reactive oxygen species (41).

Deoxyribose Degradation

Halliwell and Gutteridge have described a technique whereby treatment of the carbohydrate, deoxyribose, with iron (II) salts in phosphate buffer caused the formation of a thiobarbituric acid-reactive product that produces a chromogen (42). This provides a very convenient in vitro method for detecting hydroxyl radicals and has been further utilized as a simple assay to determine rate constants of substances that compete with deoxyribose to react with hydroxyl radicals (43).
Ferricytochrome c Reduction

In 1969, McCord and Fridovich developed a convenient technique for determining the amount of superoxide dismutase activity present in cellular suspensions (44). This method has also been utilized to determine the ability of potential antioxidants to mimic the action of SOD by competing for superoxide anion radicals with ferricytochrome c (45,46). The more effective the competition, the lower the rate of reduction of ferricytochrome c.

Spin Trapping of Free Radicals

Free radicals are paramagnetic in nature due to the unpaired electron they possess. Consequently, electron paramagnetic resonance (EPR) spectroscopy has been successfully employed to detect low concentrations of free radicals in aqueous solution. The direct detection of such paramagnetic reactive oxygen species as the superoxide anion, the hydroperoxy radical, and the hydroxyl radical is possible using EPR spectroscopy. However, the application of EPR spectroscopy to the detection of such species present within biological samples is limited by the fact that many free radicals of biological interest are highly reactive and rarely reach a high enough concentration to be detected by EPR spectroscopy.

Approximately a decade ago, an innovative technique called "spin trapping" was introduced by Janzen (47). Spin trapping overcame most of the problems of other techniques used to detect small quantities of free radicals in biological samples, such as high-energy radiolysis and high-intensity photolysis. A spin trap is a diamagnetic substituted nitrone or nitroso compound which reacts with a radical to form a radical product known as the spin adduct (Scheme F). The adduct is more stable relative to the
radical alone, in that the adduct is less reactive and therefore reaches concentrations high enough to be measured.

![Chemical structure](image)

**Scheme F: Formation of Spin Adduct**

The relative stability of the spin adduct as compared to the radical itself allows the spin adduct to accumulate in the biological system to a concentration that is detectable by EPR spectroscopy. Under favorable conditions, an EPR spectrum will be observed from which analysis of the intensity and splitting characteristics yields the identity of the radical. A direct relationship exists between the intensity of the spectral signal and the number of radicals under observation. Therefore, it is possible to use this technique and the appropriate spin trapping agent to gather information about the identity of radical species formed, as well as to quantitate the radicals being generated under varying conditions within a biological system. One of the spin traps for the detection of oxygen-centered radicals is a commercially available nitrone, 5,5'-dimethyl-1-pyroline-N-oxide (DMPO), which can form adducts with the hydroxyl radical, the hydroperoxy radical, and the superoxide anion (48). Nitrones can react with and trap a variety of radicals including oxygen-, nitrogen-, and carbon-centered free radicals, whereas nitroso compounds react mainly with carbon-centered radicals.
D. Free Radical Production by Leukocytes

Leukocytes are cells that function in vivo mainly to kill microbes and digest foreign substances which they encounter as they are distributed to the tissues via the circulatory system. Leukocyte is a broad cellular classification which includes phagocytic cell types such as, neutrophils, macrophage/monocytes, and eosinophils. Each of these cell types possesses an oxygen-dependent bactericidal mechanism in which a series of reactions leads to the production of reactive oxygen species (ROS) that, in turn, either react directly with bacteria or interact with other substances to produce additional reactive species. Under normal circumstances, this system is advantageous to the host because it is used to oxidatively kill microbes or degrade cellular debris. However, if leukocytes are inappropriately stimulated then undesirable toxic oxygen species are produced in the host which may cause oxidative tissue damage. Clinical situations which are believed to involve leukocyte-derived oxidative tissue injury include inflammation, trauma, and ischemia-reperfusion injury (49).

The oxygen-dependent bactericidal mechanism is initiated by phagocytosis or disturbances of the cell membrane that ultimately leads to the activation of the membrane-bound NADPH oxidase enzyme. The activation of the enzyme rapidly increases the oxygen consumption of the cell and has been termed the respiratory burst of the leukocyte. NADPH oxidase catalyzes the reduction of oxygen to form the superoxide anion radical within the phagolysosome. The generation of superoxide by the respiratory burst of NADPH oxidase is followed by the rapid production of additional toxic oxygen derivatives including, •OH, \( \cdot O_2^- \), and \( H_2O_2 \). The NADPH oxidase reaction requires a continuous supply of NADPH which is supplied by an increased glucose-6-phosphate metabolism through the hexose monophosphate shunt.
The oxygen-dependent bactericidal mechanism of leukocytes also involves myeloperoxidase. This enzyme uses H₂O₂, formed from the dismutation of O₂⁻⁻, and halide ions to form hypohalous acids, such as hypochlorous acid (HOCl). The HOCl is used by the leukocyte in conjunction with the other ROS to produce a series of secondary chemical reactions that destroy bacteria and other cells. An excellent overview of leukocyte function is provided by Zinkl (50).

A central issue in the study of ischemia-reperfusion injury in the lung has been determining the relative roles of leukocyte and non-leukocyte-derived oxidants in the pathogenesis of this type of injury. The initial proinflammatory event during ischemia-reperfusion injury is thought to be non-leukocyte-derived ROS. The initial production of ROS leads to subsequent adhesion and activation of leukocytes which exacerbate the injury process by releasing cytotoxic enzymes and ROS directly onto the microvascular endothelium (51-53). Adhesion of leukocytes to microvascular endothelium was shown to be a rate-limiting step in ischemia-reperfusion-induced endothelial injury by Granger (51) and ROS are believed to promote additional adhesion. In a study performed by Seibert et al. it was found that isolated, buffer-perfused rat lungs contain significant numbers of endogenous leukocytes which contribute to ischemia-reperfusion-induced microvascular injury (54). Endothelial damage gives rise to edema and increased vascular resistance which are indices of ischemia-reperfusion injury and hallmarks of the inflammatory process.
E. Taurine and Hypotaurine as Antioxidants

Taurine is a sulfur-containing β-amino acid which is ubiquitous throughout the tissues of animals.

\[
\text{H}_2\text{N-CH}_2\text{-CH}_2\text{-SO}_3\text{H}
\]

\text{Taurine}

Scheme G: Taurine

Taurine is found in high concentrations in oxidant generating cells and tissues, such as neutrophils and retina (55). The reason for the relatively high concentration of taurine in these tissues is speculated to be that taurine plays a significant role in protecting these tissues from oxidative stress. In the neutrophil, excessive hypochlorous acid (HOCl) is produced by the enzymatic oxidation of chloride ions (Cl\(^-\)) by the enzyme myeloperoxidase (56-58). HOCl is a powerful oxidant used as a biocidal agent in disinfecting public water sources. HOCl may lead to lipid peroxidation and may also directly oxidize such biologically significant substances as carbohydrates, nucleic acids, peptide linkages, and amino acids (59-60). It has been shown that taurine acts as an efficient scavenger of HOCl in biological systems (61). HOCl used to chlorinate swimming pools has been shown by Kitano and Yoshimura to cause corneal damage (62) and, in a separate study, taurine was shown to be effective in healing corneal damage caused by HOCl in rabbits (63). Nakamori et al. demonstrated the ability of taurine to protect canine erythrocytes from lysis by exposure to HOCl, lending further evidence that taurine acts as a scavenger for HOCl, a biologically important oxidant (64).

Taurine is believed to play a role in the stabilization of membranes, as demonstrated in the photoreceptor cell (65), the tapetum lucidum of the taurine-deficient cat (66), and
the cones of the photoreceptor layer of the taurine-deficient monkey (67). Huxtable and Bressler showed that taurine could counteract the damaging effects of external agents (phospholipase C and p-chloromercuribenzoate) to the intracellular membrane of skeletal muscle (68), while Kramer et al. showed a similar effect in heart muscle (69). Alvarez and Storey demonstrated that taurine and, more effectively, hypotaurine were able to protect rabbit spermatozoa from loss of motility due to spontaneous lipid peroxidation (70). The major inducing agent of lipid peroxidation in rabbit spermatozoa has been shown to be the superoxide anion species produced by the cells during aerobic incubation (71,72). This fact led Alvarez and Storey to postulate that hypotaurine may function as an intracellular scavenger that inhibits lipid peroxidation and the inactivation of superoxide dismutase by superoxide and hydrogen peroxide (72,73).

\[
\text{H}_2\text{N-CH}_2\text{-CH}_2\text{-SO}_2\text{H}
\]

Hypotaurine

**Scheme H: Hypotaurine**

In 1985, Fellman and Roth focused on the potential role of hypotaurine as a specialized antioxidant (74). They point out the potential oxidizability of the sulfinate moiety of hypotaurine as the chemical basis of its antioxidant properties and propose that hypotaurine functions as a hydroxyl radical trap in tissue (55). Specifically, they hypothesize that hydroxyl radicals generated in the tissue may react with hypotaurine to extract an electron to form the resonance-stabilized hypotaurine radical plus hydroxide ion. Two hypotaurine radicals, so generated, could unite and form a disulfone intermediate which may rearrange by an internal oxidation-reduction reaction to yield one mole of taurine and one mole of hypotaurine. If this chemistry is accurate,
hypotaurine (via the resonance-stabilized sulfinate free radical) would be a likely protector of tissues from the hydroxyl radical which is the powerful oxidant believed to be a primary initiator of lipid peroxidation in many tissues.

Milei et al. published a study in 1992 (75) that was designed to assess indirectly the free radical scavenging ability of taurine in patients with stable angina who underwent coronary artery bypass grafting. At between 1 and 3 hours prior to surgery, patients were given a 5 gram rapid intravenous infusion of taurine or a placebo injection (control). Using transmission electron microscopy and hydroperoxide-initiated chemiluminescence to assess ischemia reperfusion injury in biopsy samples taken during the pre-ischemic period and 10 minutes post-reperfusion, these authors reported the effect of the taurine treatment to be a reduction in the occurrence of reperfusion lipoperoxidation apparently generated by oxygen radical overproduction and a decrease in cell damage, as evidenced by electron microscopy.

In cases where taurine and hypotaurine have been shown to act as antioxidants, the exact mechanism by which taurine and its metabolite exert this effect remains unclear. There are conflicting opinions within the literature about taurine’s mechanism of action. Taurine has been shown to possess antioxidant properties (76-79) by various investigators, some of whom propose that taurine does this by interacting directly with the reactive species of oxygen as mentioned above and others who propose taurine acts to alter membrane function in a protective fashion by influencing calcium flux (68,69,79-86). Gordon et al. performed studies which implicated taurine as an inducer of one or more of the steps in gap junction formation and they propose that a mechanism involving calcium ions may play an important role in gap junction
formation. These authors note that taurine can easily cross the plasma membrane and speculate that taurine may intercalate into the membrane, thereby effecting the calcium ion channels, calmodulin activation, and subsequently, the calcium flux (86).

This study focused on further characterization of the antioxidant properties of taurine and hypotaurine and attempted to determine the effect of pharmaceutical intervention during the ischemic and the reperfusion period using taurine and hypotaurine, compounds believed to possess antioxidant properties.
Chapter 2

MATERIALS AND METHODS

A. Reagents

All reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

B. $H_2O_2$ Production by Macrophages

In these studies, the method of Bass et al. for the detection of $H_2O_2$ production, using the chemical $2',7'$-dichlorofluorescin diacetate (DCFH-DA) (Eastman Kodak, Rochester, NY) and polymorphonuclear leukocytes (38), was employed. This method afforded the ability to monitor a change in fluorescence intensity over a period of time at an excitation wavelength of 485 nanometers and an emission wavelength of 530 nanometers using a Cytofluor 2300 spectrofluorometer (Millipore Corporation, Bedford, MA). The increase in intensity was considered to be representative of production of $H_2O_2$ by the cells.

The cell line used in the DCFH-DA assays was the mouse monocyte-macrophage cell line RAW 264.7 (from Albeson leukemia virus transformed Balb/c mice, American Type Culture Collection, Rockville, MD). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified incubator with an atmosphere of 5% carbon dioxide and 95% air. The macrophages were grown to confluence in tissue culture flasks over a period of approximately 5 days. On the day of an experiment, the tissue culture flasks were scraped and the macrophages transferred, using sterile technique, to 24-well cell culture plates. Cells were plated on 24-well plates at $1 \times 10^6$ cells per well and allowed to adhere to the
bottom of the plate. The DMEM was aspirated from the 24-well plate and replaced with Hank's Balanced Salt Solution (HBSS) with magnesium and calcium but without phenol red. The concentration and incubation time of the DCFH-DA with the cells were chosen to be 5 μM and 25 minutes at 37°C, respectively. The concentration and incubation time used in this study were the same conditions reported in the literature by Bass et al.(38).

Phorbol esters have been shown to stimulate macrophages to produce H₂O₂ (87). The optimum level of the phorbol ester, phorbol myristate acetate (PMA), was determined as follows. After cells had been placed in 24-well plates, allowed to adhere, and incubated as described above with 5 μM DCFH-DA, a fluorescence measurement was taken (t = 0). PMA was added to the cells, immediately following the measurement at t = 0, at concentrations of 0.001, 0.01, 0.1, 1 and 10 μg/ml and measurements of fluorescence intensity were taken at 30, 45, and 60 minutes. The treatment with PMA that provided both a convenient incubation time and a high level of fluorescence above that of the control containing no PMA was selected to be used for all further experiments. PMA stimulation of macrophages was optimized at a concentration of 1 μg/ml and incubation time of 45 minutes at 37°C.

The concentrations of taurine and hypotaurine to be tested for inhibition of PMA-stimulated H₂O₂ production were first tested to determine each reagent's own ability to stimulate macrophages to produce H₂O₂. After cells had been placed in 24-well plates, allowed to adhere, and incubated as described above with 5 μM DCFH-DA, a fluorescence measurement was taken (t = 0). Taurine was added immediately following the fluorescence measurement at t = 0 at concentrations of 5, 20, 40, 120,
and 160 mM and fluorescence measurements were taken at 30, 45, and 60 minutes. Hypotaurine was added, in the same fashion, at concentrations of 0.1, 0.4, 0.8, 2.4, and 3.2 mM and fluorescence measurements were taken at the same time points. The control for each of the two reagents contained cells incubated with 5 μM DCFH-DA for 25 minutes at 37°C. The control contained no PMA and no taurine or hypotaurine.

After the determination of optimal H2O2 production by the RAW 264.7 cell line, quinidine, a reagent previously shown to inhibit H2O2 production by RAW 264.7 cells (41), was added to demonstrate that known H2O2 production inhibitors could inhibit the fluorescence being measured in the current system. Quinidine was added to the cells just prior to the DCFH-DA and incubated with the 5 μM DCFH-DA as described above at concentrations of 0.05, 0.1, 0.5, 1, and 2 mM. PMA at a final concentration of 1 μg/ml was added just after a fluorescence measurement was taken (t = 0) and the reaction mixture was incubated 45 minutes at 37°C. The fluorescence measured at t = 45 minutes for the samples containing quinidine was compared with that of a control containing 1 μg/ml PMA but no quinidine.

The effect of taurine on PMA-stimulated H2O2 production by RAW 264.7 cells was determined. Taurine was added to the cells just prior to the DCFH-DA and incubated with the 5 μM DCFH-DA as described above at concentrations of 3.3, 13, 26, 78, and 104 mM. PMA at a final concentration of 1 μg/ml was added just after a fluorescence measurement was taken (t = 0) and the reaction mixture was incubated 45 minutes at 37°C. The fluorescence measured at t = 45 minutes for the samples containing taurine was compared with that of a control containing 1 μg/ml PMA but no taurine.
The effect of hypotaurine on PMA-stimulated $\text{H}_2\text{O}_2$ production by RAW 264.7 cells was determined. Hypotaurine was added to the cells just prior to the DCFH-DA and incubated with the 5 $\mu$M DCFH-DA as described above at concentrations of 0.1, 0.4, 0.8, 2.4, and 3.2 mM. PMA at a final concentration of 1 $\mu$g/ml milliter was added just after a fluorescence measurement was taken ($t = 0$) and the reaction mixture was incubated 45 minutes at 37°C. The fluorescence measured at $t = 45$ minutes for the samples containing hypotaurine was compared with that of a control containing 1 $\mu$g/ml PMA but no hypotaurine.

C. Enzymatically-Induced Microsomal Lipid Peroxidation

Microsomal protein fractions from bovine lungs were isolated using a modification of the method of Matsubara et al. (88). Lung tissue was graciously donated by Smith Valley Meats (Rich Creek, VA). A portion of one lung lobe was removed at the time of slaughter and immediately placed in ice-cold 0.9% saline. The tissue was washed 2-3 times in fresh volumes of the saline. The average weight of the tissue was approximately 800 grams. The large blood vessels, bronchi, and outer layer of connective tissue were dissected away and discarded. Two volumes of cold, 0.32 M sucrose buffer containing 10 mM Tris, 150 mM NaCl, 1 mM MgSO$_4$, and 2 mM EDTA, pH 7.4, were added to the remaining 250 grams of tissue. All manipulations of the lung tissue were done at 4°C.

The mixture was homogenized thoroughly using an industrial strength Waring Blender at the highest setting for 2-3 minutes. The homogenate was filtered through one layer of gauze cloth using mild agitation with a Teflon spatula and one additional volume of 0.32 M sucrose buffer. The final volume was recorded, divided into centrifuge tubes
and sedimented at 1000 x g for 10 minutes in a refrigerated centrifuge. The supernatant fraction was decanted into ultra-centrifuge tubes and centrifuged at 10,000 x g for 10 minutes. The 10,000 x g supernatant fraction was collected and centrifuged at 16,000 x g for 10 minutes. The 16,000 x g supernatant was collected and centrifuged for 60 minutes at 105,000 x g. The microsome-enriched sediment fraction was collected, resuspended in the original volume of ice-cold 1 mM Tris-HCl buffer, pH 7.6, and sedimented again at 105,000 x g for 60 minutes. All centrifugation was performed at 4°C. The final supernatant fraction was discarded and all microsomal fractions were resuspended and combined in a final volume of 30 ml of 0.1 M Tris-HCl buffer, pH 7.6, if they were to be used immediately. When not used immediately, the microsomal fractions were not resuspended; instead, they were frozen as pellets without buffer at -70°C and used within 7 days of their isolation. Isolation was performed two times.

Once the microsomal protein-enriched pellets were resuspended in 0.1 M Tris-HCl buffer, pH 7.6, the suspension was homogenized very gently using a Teflon-glass Dounce homogenizer and motor-driven pestle (Fisher Scientific) at setting 4-5 for 5 strokes at 4°C. The method of Bradford (89) was used to determine the protein concentration of the homogeneous protein suspension employing bovine serum albumin as the standard.

An in vitro model of enzymatically-induced lipid peroxidation was developed using the technique described by both Bernheim et al. (90) and Wills (91). This technique is one whereby, under acidic conditions, lipid peroxidation products react with thiobarbituric acid to form a colored product spectrophotometrically detectable at a wavelength of 535
nanometers (nm). In these studies, a 1 ml reaction volume contained the following: 1.5 mg of microsomal protein, 50 µM FeCl₃, 1 mM ADP, and 0.2 mM NADPH in 0.1 M Tris-HCl buffer, pH 7.6. The FeCl₃ and ADP solutions were mixed together 1 day prior to the experiment. Lipid peroxidation was initiated at 37°C with the addition of NADPH and terminated 15 minutes later with the addition 2 ml of a 0.5% thiobarbituric acid (TBA) solution containing 2% trichloroacetic acid (TCA). The mixture was boiled for 10 minutes to allow the chromogen to form, and then cooled to room temperature prior to the addition of 3 ml of chloroform to precipitate organic lipid debris that might interfere with the measurement of absorbance at 535 nm. The samples were vortexed for 30 seconds and centrifuged at 800 x g for 10 minutes to separate the organic and aqueous phases. The aqueous layer was aspirated and placed in a cuvette to have its absorbence measured at 535 nm using a UV-160 spectrophotometer (Shimadzu).

After establishing a consistent level of MDA formation associated with the amount of protein being utilized within the current system, known scavengers of reactive oxygen species (oxy-radicals) were added to the system to determine that the lipid peroxidation activity present could be inhibited by oxy-radical scavengers. A single concentration of each oxy-radical scavenger was selected and tested. The reagents chosen were mannitol (10 mM), thiourea (10 mM), manganese chloride (MnCl₂, 10 mM), and diethylenetriamine pentaacetic acid (DETAPAC, 0.8 mM). Each of these reagents was added to a reaction mixture prior to the addition of NADPH as described above and compared to a "positive control" that only contained the reaction mixture ingredients without oxy-radical scavengers. The percentage of the positive control was calculated as:
(Abs535 experimental ÷ Abs535 control) * 100 = % of positive control

The effect of taurine was tested in this system. Taurine was added to reaction mixtures prior to the addition of NADPH as described above at concentrations of 5, 10, 20, 40, and 120 mM. The effect of taurine was determined by comparing each taurine sample to a positive control containing all reaction mixture ingredients, but no taurine. A percentage of positive control value was calculated.

The effect of hypotaurine was tested in this system. Hypotaurine was added to a reaction mixture prior to the addition of NADPH as described above at concentrations of 2, 4, 6, 8, and 10 mM. The effect of hypotaurine was determined by comparing each hypotaurine sample to a positive control containing all reaction mixture ingredients, but no hypotaurine. A percentage of positive control value was calculated.

D. Deoxyribose Degradation

Hydroxyl radicals (·OH) are known to damage the sugar deoxyribose yielding a thiobarbituric acid (TBA) reactive chromogen that can be monitored spectrophotometrically at 535 nm (42). Hydroxyl radicals were generated chemically in a Fenton-type system in the presence of deoxyribose:

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

The total volume of the reaction mixture was 1.2 ml and contained the following reagents: 30 μM EDTA, 25 μM FeCl₃, 100 μM ascorbate, 1 mM H₂O₂, and 2.8 mM deoxyribose in 20 mM KH₂PO₄-KOH buffer, pH 7.4. Deoxyribose degradation was
initiated at 37°C with the addition of deoxyribose to the reaction mixture and terminated 60 minutes later with the addition of 2 ml of a solution containing 1.4% TCA and 0.5% TBA. The samples were heated at 80°C for 30 minutes to allow the development of the TBA-reactive product, malondialdehyde (MDA). The tubes were allowed to cool to room temperature before the absorbances were measured at 535 nm using a Shimadzu UV-160 spectrophotometer.

After establishing a consistent level of MDA formation associated with the amount of deoxyribose being utilized within the current system, known scavengers of oxy-radicals were added to the system to determine that the deoxyribose degradation present was able to be inhibited by oxy-radical scavengers. A single concentration of each oxy-radical scavenger was selected and tested. The reagents chosen were mannitol (10 mM), thiourea (10 mM), MnCl₂ (10 mM), and DETAPAC (0.8 mM). Each of these reagents was added to a reaction mixture prior to the addition of deoxyribose as described above and compared to a positive control that only contained the reaction mixture ingredients. A percentage of positive control value was calculated.

The effect of taurine was tested in this system. Taurine was added to a reaction mixture prior to the addition of deoxyribose as described above at concentrations of 5, 10, 20, 40, and 100 mM. The effect of taurine was determined by comparing each taurine sample to a positive control containing all reaction mixture ingredients, but no taurine. A percentage of positive control value was calculated.

The effect of hypotaurine was tested in this system. Hypotaurine was added to a reaction mixture prior to the addition of deoxyribose as described above at
concentrations of 2, 4, 6, 8, and 10 mM. The effect of hypotaurine was determined by comparing each hypotaurine sample to a positive control containing all reaction mixture ingredients, but no hypotaurine. A percentage of positive control value was calculated.

**E. Ferricytochrome c Reduction**

The method of McCord and Fridovich (44) was used to determine the effect of taurine and hypotaurine on the reduction of ferricytochrome c by superoxide radicals. Commercially available SOD was used to prepare a solution of 100 units of activity/ml (31 µg/ml). One unit of SOD is defined as the amount of SOD required to reduce the rate of reduction of ferricytochrome c by 50%. The SOD solution was used to verify O$_2^-$-dependent reduction of ferricytochrome c. Reaction mixtures of 1 ml total volume contained final concentrations of the following reagents: 5 x 10$^{-5}$ M xanthine, 52 µg/ml xanthine oxidase, and 10$^{-5}$ M cytochrome c in 0.05 M potassium phosphate buffer with 10$^{-4}$ M EDTA, pH 7.8. The reagents for these experiments were kept at 25°C and exposure to light was limited. The reaction was initiated by the addition of xanthine oxidase. The reduction of ferricytochrome c was monitored using the kinetic program of the Shimadzu UV-160 spectrophotometer at a wavelength of 550 nm. The reduction of ferricytochrome c was linear for at least 5 minutes and a final concentration of 0.28 µg/ml of commercially prepared SOD inhibited the reduction rate by 50%, indicating that the reduction of ferricytochrome c was O$_2^-$-dependent.

The effect of taurine was tested in this system. Taurine was added to a reaction mixture at the same time as the xanthine oxidase as described above at concentrations of 5, 10, 20, 38, and 107 mM. The percentage of inhibition was calculated by
comparing the rate of reduction determined for the taurine sample to that determined for a control monitored over the same period of time but containing no taurine.

The effect of hypotaurine was tested in this system. Hypotaurine was added to a reaction mixture at the same time as the xanthine oxidase as described above at concentrations of 2, 4, 6, 8, and 10 mM. The effect of hypotaurine was determined by comparing each hypotaurine sample to a control monitored over the same time period, containing all reaction mixture ingredients, but no hypotaurine. The percentage of inhibition value was calculated by comparing the rate of reduction determined for the hypotaurine sample to that determined for a control monitored over the same period of time but containing no hypotaurine.

F. Spin Trapping Studies

Well established chemical systems for the generation of superoxide anion radical and hydroxyl radical in vitro were used in this study. The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used to detect DMPO-OH, the spin adduct of the spin trap plus the hydroxyl radical. Hydroxyl radicals were generated chemically in a Fenton-type system:

\[ \text{Fe}^{3+} + \text{Ascorbate} \rightarrow \text{Fe}^{2+} + \text{Ascorbate}\cdot \]

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

The reaction volume was 0.1 ml and contained the following reagents: 60 μM EDTA, 50 μM FeCl₃, 100 μM ascorbate, 2 mM H₂O₂, and 52 mM DMPO in 100 mM NaCl/25 mM NaHCO₃ buffer, pH 7.0. The addition of H₂O₂ was used to start the
reaction. The DMPO-OH adduct was recorded immediately as the characteristic 1:2:2:1 electron paramagnetic resonance (EPR) spectrum with a split constant of approximately 15 gauss and was relatively stable for 1 hour. The EPR measurements were made at room temperature on a Bruker D-200 X-band spectrometer (IBM Instruments) using a magnetic field modulation frequency of 100 K Hz, modulation amplitude of 1.0 G, time constant of 0.64 s, scan rate of 500 s, center field setting of 3483 G; and a receiver gain of $1 \times 10^6$. The hydroxyl radical scavenger, thiourea (6, 12, and 18 mM), was used to determine if the signal detected was that of the hydroxyl radical. DMPO was tested alone to insure its purity.

Taurine concentrations tested in this system were 5, 50, 75, 100, 200, and 300 mM. Taurine was added just after the DMPO and prior to the addition of $H_2O_2$. Hypotaurine was tested at 10, 20, and 30 mM and also was added just after the DMPO and prior to the addition of $H_2O_2$. The peak-to-peak height of the second line from the lower field was determined to be the most consistent among 20 repetitions of the control sample, therefore it was used as the reference peak to determine "% inhibition" values.

The spin trap 5,5,-dimethyl-1-pyrroline-N-oxide (DMPO) also was used to detect the DMPO-OOH spin adduct which is the product of reaction of DMPO and $O_2^-$. Generation of $O_2^-$ was achieved using a 0.155 ml reaction volume containing $1 \times 10^{-4}$ M xanthine, 1 mg/ml xanthine oxidase and 210 mM DMPO in 35% dimethyisulfoxide (DMSO) and 0.05 M potassium phosphate buffer with $10^{-4}$ M EDTA, pH 7.8. The DMPO-OOH adduct was measured at 75 seconds after the addition of xanthine oxidase to start the reaction. The EPR spectrum was stable for less than 8 minutes but
demonstrated the pattern characteristic of the DMPO-OOH adduct. The EPR measurements were made at room temperature on a Bruker D-200 X-band spectrometer using a magnetic field modulation frequency of 100 K Hz, modulation amplitude of 0.710 G, time constant of 1.25 s, scan rate of 500 s, center field setting of 3483 G; and a receiver gain of $2 \times 10^6$. SOD was added to the reaction mixture prior to the addition of xanthine oxidase at approximately 6.45 units of activity/ml. This control was included to determine that the spectrum obtained was that of the superoxide-DMPO adduct.

Taurine concentrations tested in this system were 5, 50, 77, 148, and 193 mM. Taurine was added just after the DMPO and prior to the xanthine oxidase. Hypotaurine was tested at 10, 20, and 30 mM and also was added just after the DMPO and prior to the xanthine oxidase. The peak-to-peak height of the second line in the lower field was determined to be the most consistent among 16 repetitions of the control sample, therefore it was used as the reference peak to determine "% inhibition" values.

**G. Ex vivo Model of Ischemia-Reperfusion Injury**

In these experiments six, male, Sprague-Dawley rats weighing between 300-500 grams were used per experimental group. Each animal was anesthetized using 0.1 ml of sodium pentobarbital (64.8 mg/ml) per 100 grams of body weight via an intraperitoneal injection. After the animal reached the appropriate plane of anesthesia, a tracheostomy was performed such that the animal could be ventilated using a Harvard rodent ventilator at 60 strokes per minute, at tidal volume of 2.3 to 3 ml, and a positive end expiratory pressure of 2.5 centimeters of water. A gas mixture of 5% carbon
dioxide and 95% air (Industrial Gas Co., Radford, VA.) was used to ventilate the animals. A median sternotomy was performed; into the right ventricle a 0.2 ml injection of heparin (200 IU) was made and then cannulae placed into the pulmonary artery and the left ventricle to establish a perfusion circuit.

Following stabilization of the cannulae, the heart, lungs, and mediastinal structures were removed en bloc and placed in a glass humidified chamber maintained at 37°C. The perfusion circuit was maintained using a peristaltic pump. The flow rate was constant at 0.5 ml/minute/gram body weight. The perfusion buffer was Krebs-Henseleit buffer containing: 118 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO₄, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 1.90 mM CaCl₂, 11.1 mM glucose, and 4% bovine serum albumin. The temperature and pH of the buffer were constantly monitored and maintained at 37°C (plus or minus 1 degree) and 7.4 (plus or minus 0.05 pH units), respectively. The first 50 ml of the perfusion buffer through the lung were discarded to remove circulating blood elements. Subsequently, a 50 ml volume of buffer was used to establish the perfusion circuit.

Mechanical function of the lung during the procedure was assessed by several parameters monitored throughout the experiment. Parameters included: the pulmonary artery pressure using a blood pressure transducer (BPLR-0111, World Precision Instruments, New Haven, CT) connected via tubing to the perfusion circuit; the peak airway pressure monitored using a PNEU-01 pressure transducer (World Precision Instruments, New Haven, CT) connected to the ventilation tubing; and the weight of the preparation using a Fort-250 linear force transducer (World Precision Instruments,
New Haven, CT) from which the entire preparation was suspended into the humidified chamber. All transducers were calibrated prior to use.

Data collection from the transducers was accomplished by a MacLab system (World Precision Instruments, New Haven, CT) including a transbridge amplifier, an uninterruptable power supply, a MacLab 4, and a Macintosh SE computer equipped with Chart® software.

The procedure for inducing ischemia was initiated after 10 minutes of perfusion to allow the parameters to stabilize. Ventilation was stopped on the downward stroke of the ventilator (to allow slight inflation of the lungs) followed by cessation of perfusion for 60 minutes (ischemia). The slight inflation of the lungs was necessary for ease of re-inflation after ischemia. Reperfusion was initiated at the end of 60 minutes of ischemia by ventilating for one full minute, then gradually adjusting the flow rate back to that of 0.05 ml/minute/gram body weight over the first 5 minutes of reperfusion. The reperfusion period was 30 minutes.

Assessment of lung damage was made on the basis of three indices: 1) wet-to-dry weight ratio (an index of edema formation), 2) change in pulmonary artery pressure, and 3) change in peak airway pressure. Immediately following the conclusion of reperfusion, the lungs were dissected from the heart, trachea, and large bronchus structures. The lungs were weighed (wet weight) and placed in a convection oven at 120°C for 72 hours (until no further weight loss was detected). At that time, a dry weight was determined and the wet-to-dry weight ratio calculated. The ratios for the
experimental lungs (taurine 5 and 10 mM) were compared to those of the injured and uninjured controls.

For the purpose of data comparison, pulmonary artery pressure (P_a) was observed among the groups during the initial 10 minutes of perfusion (pre-ischemic) and for the last 25 minutes of reperfusion (post-ischemic). A percentage change was calculated by comparing the mean of the pre-ischemic P_a values with the mean of the post-ischemic P_a values. Peak airway pressure (P_{aw}) over the first 10 minutes of pre-ischemia was compared with the full 30 minutes of post-ischemic ventilation and a percentage change calculated. The various concentrations of taurine (5 and 10 mM) and hypotaurine (2 and 4 mM) tested in these experiments were present from the beginning of perfusion.

**H. Statistical Analysis**

All results are expressed as means ± SEM. Statistical analysis was performed on the H_2O_2 production, lipid peroxidation, and ferricytochrome c data using a Student's t-test software package (Epistat User Supported Software, Round Rock, TX). All experiments were repeated three times and each data point was done in triplicate within each assay (n = 9). Each experimental sample was compared to the control for that particular experiment. Statistically significant differences between a sample and the control were defined by a p-value ≤ 0.05. Statistical analysis was performed on the deoxyribose degradation data using a Student's t-test software package as described above except that all experiments were repeated four times and each data point was done in triplicate within each assay (n = 12). Statistical analysis of the ex vivo lung data was performed using SAS System for Elementary Statistical Analysis (SAS Institute Inc., Cary, NC). A one-way analysis of variance (ANOVA) was performed to
determine statistically significant differences between the experimental groups (uninjured controls, injured controls, taurine 5 mM, and taurine 10 mM). Subsequently, Fisher’s Least Significant Difference multiple comparison test was performed to determine which group means differed from the others. The significance level used was $\alpha = 0.05$ and a $p$ value of $p \leq 0.05$ was considered to be statistically significant.
Chapter 3

RESULTS

A. Effect of Taurine and Hypotaurine on $H_2O_2$ Production by Macrophages

The generation of $H_2O_2$ in PMA-stimulated RAW 264.7 macrophage cells, employing 5 $\mu$M 2',7'-dichlorofluorescin diacetate (DCFH-DA), was monitored as a function of PMA concentration over time. The optimum concentration of the phorbol ester, PMA, was determined to be 1 $\mu$g/ml (Figure 1). A 60 minute incubation time of 1 $\mu$g/ml PMA with the macrophages yielded the maximum measurement of fluorescence above background fluorescence present in the non-PMA control detected in this experiment. None of the experimental concentrations of PMA tested were statistically different ($p > 0.05$) from the non-PMA control at the beginning of incubation ($t = 0$). The 0.001 $\mu$g/ml PMA was not statistically different ($p > 0.05$) from the non-PMA control at 30 minutes of incubation while all higher concentrations were statistically different ($p \leq 0.05$) from the control at all time points recorded after $t = 0$.

The effects of taurine and hypotaurine on the production of hydrogen peroxide by the RAW 264.7 macrophage cells were first tested in the non-PMA system. As shown in Figure 2 and Figure 3, neither taurine nor hypotaurine were able to produce significant ($p > 0.05$) amounts of $H_2O_2$ above that of the negative control.
Figure 1. Stimulation of RAW 264.7 macrophage cells to produce hydrogen peroxide by phorbol myristate acetate (PMA). RAW 264.7 cells were plated at 1 x 10^6 cells / ml in HBSS. DCFH-DA was added to the cells at a final concentration of 5 μM and incubated at 37°C for 25 minutes as indicated by the method of Bass et al. described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. Means with a "*" above them were significantly different (p ≤ 0.05) from the control containing no PMA. The effect of adding 0.001, 0.01, 0.1, 1.0, and 10 μg/ml of PMA was determined by measuring the fluorescence intensity just before the addition of PMA (t = 0) and at 30, 45, and 60 minutes after its addition. The control (0 μg/ml PMA), to which PMA containing samples were compared, contained 1 x 10^6 cells / ml in HBSS and 5 μM DCFH-DA and was monitored at the same time points as the PMA samples. PMA at 0.001 μg/ml was not statistically different (p > 0.05) from the control at any time point. PMA at 0.01 μg/ml was not statistically different (p > 0.05) from the control at t = 0 or at t = 30 minutes but was significantly different (p ≤ 0.05) from the control at t = 45 and t = 60 minutes. PMA at concentrations of 0.1, 1, and 10 μg/ml were not statistically different from the control at t = 0 but were statistically different (p ≤ 0.05) from the control at all other time points. Significant fluorescence (H_2O_2 production) above that of the control was obtained using 1 μg/ml PMA and an incubation time of 45 minutes.
Figure 2. Effect of taurine on RAW 264.7 macrophage cells. RAW 264.7 cells were plated at 1 x 10^6 cells / ml in HBSS. DCFH-DA was added to the cells at a final concentration of 5 μM and incubated at 37°C for 25 minutes as indicated by the method of Bass et al. described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. The effect of adding 5, 20, 40, 120, and 160 mM taurine was determined by measuring the fluorescence intensity just before the addition of taurine (t = 0) and at 30, 45, and 60 minutes after its addition. The control (0 mM taurine), to which taurine-containing samples were compared, contained 1 x 10^6 cells / ml in HBSS and 5 μM DCFH-DA and was monitored at the same time points as the taurine-containing samples. Taurine (5-160 mM) demonstrated no significant ability (p > 0.05) to stimulate macrophages to produce H_2O_2 at any concentration tested.
Figure 3. Effect of hypotaurine on RAW 264.7 macrophage cells. RAW 264.7 cells were plated at 1 x 10^6 cells / ml in HBSS. DCFH-DA was added to the cells at a final concentration of 5 μM and incubated at 37°C for 25 minutes as indicated by the method of Bass et al. described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. The effect of adding 0.1, 0.4, 0.8, 2.4, and 3.2 mM hypotaurine was determined by measuring the fluorescence intensity just before the addition of hypotaurine (t = 0) and at 30, 45, and 60 minutes after its addition. The control (0 mM hypotaurine), to which hypotaurine-containing samples were compared, contained 1 x 10^6 cells / ml in HBSS and 5 μM DCFH-DA and was monitored at the same time points as the hypotaurine samples. Hypotaurine (0.1-3.2 mM) demonstrated no significant ability (p > 0.05) to stimulate macrophages to produce H₂O₂ at any concentration tested.
Quinidine, a Class I antiarrhythmic agent, was shown to inhibit 50% of optimum hydrogen peroxide production in RAW 264.7 macrophages at a concentration of 100 μM in a previous study performed by Das and Misra using the above techniques (41). As shown in Figure 4, significant inhibition of the hydrogen peroxide production was achieved at concentrations of 0.5, 1, and 2 mM quinidine (p ≤ 0.05). This control was necessary to validate the present technique.

The addition of 3.3 mM taurine to the PMA-stimulated cells produced no significant effect (p > 0.05) on the production of hydrogen peroxide. However, the addition of higher concentrations of taurine (13, 26, 78, or 104 mM) produced a significant increase (p ≤ 0.05) in hydrogen peroxide production above that of the control (Figure 5). Hypotaurine (0.1-2.4 mM) produced no significant effect (p > 0.05) on the production of hydrogen peroxide by the RAW 264.7 macrophages. However, the addition of 3.2 mM hypotaurine produced a significant increase (p ≤ 0.05) in H₂O₂ production above that of the control (Figure 6).
Figure 4. Effect of quinidine on PMA-stimulated H$_2$O$_2$ production in RAW 264.7 macrophages. RAW 264.7 cells were plated at 1 x 10$^6$ cells/ml in HBSS. DCFH-DA was added to the cells at a final concentration of 5 μM and incubated at 37°C for 25 minutes as indicated by the method of Bass et al. described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. Means with an "*" above them were determined to be significantly different (p ≤ 0.05) when compared to the control. The effect of adding 0.05, 0.1, 0.5, 1, and 2 mM quinidine on the stimulation of macrophages by PMA was determined. Quinidine was added just prior to the DCFH-DA and incubated as mentioned above. PMA at a final concentration of 1 μg/ml was added to the reaction mixture and incubated at 37°C for 45 minutes. At 45 minutes, the fluorescence intensity was measured and compared to that of a control (0 mM quinidine) which contained 5 μM DCFH-DA, 1 x 10$^6$ cells, and 1 μg/ml PMA. Quinidine demonstrated significant inhibition (p ≤ 0.05) of H$_2$O$_2$ production by macrophages at final concentrations of 0.5, 1, and 2 mM.
Figure 5. Effect of taurine on PMA-stimulated $\text{H}_2\text{O}_2$ production in RAW 264.7 macrophages. RAW 264.7 cells were plated at $1 \times 10^6$ cells/ml in HBSS. DCFH-DA was added to the cells at a final concentration of 5 $\mu$M and incubated at 37°C for 25 minutes as indicated by the method of Bass et al. described under "Materials and Methods". Each data point represents the mean ± SEM with $n = 9$. Means with a "∗" above them were found to be significantly different ($p \leq 0.05$) when compared to the control. The effect of adding 3.3, 13, 26, 78, and 104 mM taurine on the stimulation of macrophages by PMA was determined. Taurine was added just prior to the DCFH-DA and incubated as mentioned above. PMA at a final concentration of 1 $\mu$g/ml was added to the reaction mixture and incubated at 37°C for 45 minutes. At 45 minutes, the fluorescence intensity was measured and compared to that of a control (0 mM taurine) which contained 5 $\mu$M DCFH-DA, $1 \times 10^6$ cells, and 1 $\mu$g/ml PMA. Taurine demonstrated no significant effect ($p > 0.05$) on $\text{H}_2\text{O}_2$ production by macrophages at a final concentration of 3.3 mM. At 13, 26, 78, and 104 mM taurine a significant augmentation ($p \leq 0.05$) of $\text{H}_2\text{O}_2$ production by macrophages above that of the control was seen.
Figure 6. Effect of hypotaurine on PMA-stimulated H$_2$O$_2$ production in RAW 264.7 macrophages. RAW 264.7 cells were plated at 1 x 10$^6$ cells / ml in HBSS. DCFH-DA was added to the cells at a final concentration of 5 µM and incubated at 37°C for 25 minutes as indicated by the method of Bass et al. described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. Means with a "*" above them were shown to be significantly different (p ≤ 0.05) when compared to the control. The effect of adding 0.1, 0.4, 0.8, 2.4, and 3.2 mM hypotaurine on the stimulation of macrophages by PMA was determined. Hypotaurine was added just prior to the DCFH-DA and incubated as mentioned above. PMA at a final concentration of 1 µg/ml was added to the reaction mixture and incubated at 37°C for 45 minutes. At 45 minutes, the fluorescence intensity was measured and compared to that of a control (0 mM hypotaurine) which contained 5 µM DCFH-DA, 1 x 10$^6$ cells, and 1 µg/ml PMA. Hypotaurine (0.1, 0.4, 0.8, and 2.4 mM) demonstrated no significant effect (p > 0.05) on H$_2$O$_2$ production by macrophages. At a concentration of 3.2 mM, hypotaurine was shown to significantly (p ≤ 0.05) augment H$_2$O$_2$ production.
B. Effect of Taurine and Hypotaurine on

Enzymatically-Induced Microsomal Lipid Peroxidation

Lipid peroxidation of bovine lung microsomes was induced by incubating the lung microsomes with 200 μM NADPH and 1 mM ADP-chelated ferric chloride. Production of TBA-reactive MDA was considered as an index of lipid peroxidation. These concentrations of reagents were found to be optimum for maximum color production (absorbance at 535 nm). A linear rate of MDA production was observed with incubation time and reached a maximum after 10 minutes. Several known inhibitors of microsomal lipid peroxidation were employed to verify the technique. Thus, as shown in Figure 7, mannitol (10 mM), MnCl₂ (10 mM), thiourea (10 mM), and DETAPAC (0.8 mM) inhibited 4, 22, 16, and 87% of the lipid peroxidation present in the positive control, respectively. These data are consistent with results published previously (92-96).

The effect of taurine and hypotaurine was tested in this system. Pre-incubation with taurine (5-120 mM) or hypotaurine (2-10 mM) had no significant effect (p > 0.05) on microsomal lipid peroxidation (Figure 8 and Figure 9).
Figure 7. Effect of known oxy-radical scavengers on bovine lung microsome lipid peroxidation. Microsomal protein fractions were collected from bovine lungs, protein concentration was determined, and lipid peroxidation was induced as described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. Means with an "*" above them were found to be significantly different (p ≤ 0.05) when compared to the control. The positive control, to which the samples containing known oxy-radical scavengers were compared, contained the following reagents in a 1 ml reaction volume: 1.5 mg of microsomal protein, 50 μM FeCl₃, 1 mM ADP, and 0.2 mM NADPH in 0.1 M Tris-HCl buffer, pH 7.6. The known oxy-radical scavengers tested in this system were as follows (final concentration): mannitol (10 mM), MnCl₂ (10 mM), thiourea (10 mM), and DETAPAC (0.8 mM). The addition of the NADPH was used to start the reaction. The reaction mixtures were treated as described under "Materials and Methods." Each of the oxy-radical scavengers was shown to significantly inhibit (p ≤ 0.05) color production. Mannitol, thiourea, MnCl₂, and DETAPAC inhibited the enzymatically-induced lipid peroxidation by 4, 16, 22 and 87%, respectively.
Figure 8. Effect of taurine on bovine lung microsome lipid peroxidation. Microsomal protein fractions were collected from bovine lungs, protein concentration was determined, and lipid peroxidation was induced as described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. The positive control, to which the samples containing taurine were compared, contained the following reagents in a 1 ml reaction volume: 1.5 mg of microsomal protein, 50 μM FeCl₃, 1 mM ADP, and 0.2 mM NADPH in 0.1 M Tris-HCl buffer, pH 7.6. Taurine was added at 5, 10, 20, 40, and 120 mM. The addition of the NADPH was used to start the reaction. The reaction mixtures were treated as described under "Materials and Methods." Taurine (5-120 mM) demonstrated no statistically significant (p > 0.05) protective effect against lipid peroxidation at any concentration tested.
Figure 9. Effect of hypotaurine on bovine lung microsome lipid peroxidation. Microsomal protein fractions were collected from bovine lungs, protein concentration was determined, and lipid peroxidation was induced as described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. The positive control, to which the samples containing hypotaurine were compared, contained the following reagents in a 1 ml reaction volume: 1.5 mg of microsomal protein, 50 μM FeCl₃, 1 mM ADP, and 0.2 mM NADPH in 0.1 M Tris-HCl buffer, pH 7.6. Hypotaurine was added at 2, 4, 6, 8, and 10 mM. The addition of the NADPH was used to start the reaction. The reaction mixtures were treated as described under "Materials and Methods." Hypotaurine (2-10 mM) demonstrated no statistically significant (p > 0.05) protective effect against lipid peroxidation at any concentration tested.
C. Effects of Taurine and Hypotaurine on Hydroxyl Radical Mediated-Deoxyribose Degradation

Degradation of the sugar deoxyribose by hydroxyl radicals was monitored via the formation of a TBA-reactive chromogen by measuring absorbance at 535 nm using the Fenton-type system:

\[
\begin{align*}
\text{Fe}^{3+} + \text{Ascorbate} \quad \rightarrow \quad & \text{Fe}^{2+} + \text{Ascorbate} \cdot \\
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \quad \rightarrow \quad & \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}
\end{align*}
\]

An increased absorbance measurement was indicative of an increased degree of degradation of deoxyribose. Several known hydroxyl radical scavengers were utilized in this system to provide assurance that the production of the chromogen in the assay was indeed hydroxyl radical dependent. The •OH generated in this system is equally accessible to deoxyribose (the detector molecule) and to any other added •OH scavenger. Thus, DETAPAC (0.8 mM), MnCl₂ (10 mM), mannitol (10 mM) and thiourea (10 mM) inhibited 33, 39, 67 and 97% of the deoxyribose degradation, respectively (Figure 10).

Taurine and hypotaurine were tested in the deoxyribose degradation system at various concentrations to determine the ability of each to protect the sugar from being degraded. As shown in Figure 11, taurine significantly (p ≤ 0.05) inhibited deoxyribose degradation. As little as 5 mM taurine inhibited 66% of the reaction as compared to the control. Taurine (100 mM) was able to inhibit up to 79% of the deoxyribose degradation present in the control, however, when a competition plot was
attempted (43) the inhibition proved to be non-linear and, therefore, not dosage dependent.

Hypotaurine demonstrated significant \((p \leq 0.05)\) inhibition of degradation at all concentrations \((2-10\) mM\) tested (Figure 12), and \(8-10\) mM virtually prevented the degradation process. Hypotaurine demonstrated dosage dependent inhibition of the degradation of deoxyribose and when plotted as described by Halliwell et al. (43) the hypotaurine data conformed to a linear competition plot \((R\) value \(= 0.99458)\). The slope of the line was utilized to calculate a rate constant for the reaction of hypotaurine with the hydroxyl radical by inserting the value for slope into the equation \(k_{hyp} = \text{slope} \times k_{DR} \times [DR] \times A\). The value of \(3.1 \times 10^{9}\) M\(^{-1}\) s\(^{-1}\) used for \(k_{DR}\) was that reported by Halliwell et al.. The value of \([DR]\) represents the concentration of deoxyribose used in the assay and was \(2.8\) mM. The value of \(A\) was determined using the y intercept of the competition plot \((1/ A = y\) intercept\). The second order rate constant for hypotaurine was determined to be \(k = 2.64 \times 10^{7}\) M\(^{-1}\)s\(^{-1}\).
Figure 10. Effect of known oxy-radical scavengers on the degradation of deoxyribose. The experimental conditions were as described in "Materials and Methods". Each data point represents the mean ± SEM with n = 12. Means with an "*" above them were found to significantly different (p ≤ 0.05) when compared to the control. Reaction mixtures contained (in 1.2 ml) 30 µM EDTA, 25 µM FeCl₃, 100 µM ascorbate, 1 mM H₂O₂, and 2.8 mM deoxyribose in 20 mM KH₂PO₄-KOH buffer, pH 7.4. The positive control contained these reagents alone, whereas, the other samples also contained one of the following: 10 mM mannitol, 10 mM MnCl₂, 10 mM thiourea, or 0.8 mM DETAPAC in addition to these reagents. All oxy-radical scavengers were shown to significantly inhibit (p ≤ 0.05) deoxyribose degradation. DETAPAC MnCl₂, mannitol, and thiourea inhibited 33, 39, 67, and 93% of the degradation of deoxyribose compared to the positive control, respectively.
Figure 11. Effect of taurine on the degradation of deoxyribose. The experimental conditions were as described in "Materials and Methods". Each data point represents the mean ± SEM with n = 12. Means with an "*" above them were found to be significantly different (p ≤ 0.05) when compared to the control. Reaction mixtures contained (in 1.2 ml) 30 μM EDTA, 25 μM FeCl₃, 100 μM ascorbate, 1 mM H₂O₂, and 2.8 mM deoxyribose in 20 mM KH₂PO₄-KOH buffer, pH 7.4. The positive control contained these reagents alone, whereas, the other samples also contained taurine at concentrations of 5, 10, 20, 40, or 120 mM in addition to these reagents. The addition of each concentration of taurine tested was shown to significantly inhibit (p ≤ 0.05) deoxyribose degradation. Taurine at 5, 10, 20, 40, and 100 mM inhibited 66, 74, 59, 72, and 79% of the degradation of deoxyribose compared to the positive control.
Figure 12. Effect of hypotaurine on the degradation of deoxyribose. The experimental conditions were as described in "Materials and Methods". Each data point represents the mean ± SEM with n = 12. Means with an "*" above them were found to be significantly different (p ≤ 0.05) when compared with the control. Reaction mixtures contained (in 1.2 ml) 30 μM EDTA, 25 μM FeCl₃, 100 μM ascorbate, 1 mM H₂O₂, and 2.8 mM deoxyribose in 20 mM KH₂PO₄-KOH buffer, pH 7.4. The positive control contained these reagents alone, whereas, the other samples also contained hypotaurine at a concentration of 2, 4, 6, 8, or 10 mM in addition to these reagents. The addition of each concentration of hypotaurine tested was shown to significantly inhibit (p ≤ 0.05) deoxyribose degradation. Hypotaurine at 2, 4, 6, 8, and 10 inhibited 69, 82, 89, 93, and 94% of the degradation of deoxyribose compared to the positive control.
D. Effect of Taurine and Hypotaurine on

Superoxide Radical-Mediated Ferricytochrome c Reduction

Superoxide radicals are known to be produced when xanthine oxidase acts on xanthine in the presence of molecular oxygen. The \( \text{O}_2^- \) so generated can reduce ferricytochrome c, and this has been used as a convenient assay to quantitate \( \text{O}_2^- \) (44,97). Taurine and hypotaurine were added to this system and their respective abilities to inhibit the reduction of ferricytochrome c by the superoxide anion radical were determined. As shown in Figure 13, taurine exhibited significant inhibition (\( p \leq 0.05 \)) of ferricytochrome c reduction in a dose-dependent manner. Thus, 10, 20, 38, and 107 mM taurine inhibited this reaction by 4, 10, 15, and 34%, respectively. At a 5 mM concentration taurine was found to have no significant effect (\( p > 0.05 \)) on the reduction of ferricytochrome c. Hypotaurine, on the other hand, inhibited the reduction of ferricytochrome c significantly (\( p \leq 0.05 \)) but modestly. Thus, 6, 8, and 10 mM hypotaurine inhibited the reaction by 5, 2, and 6%, respectively (Figure 14).
Figure 13. The effect of taurine on ferricytochrome c reduction by superoxide radicals. Experimental conditions were as described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. Means with a "**" above them were found to be significantly different (p ≤ 0.05) when compared with the control. Taurine was added to a 1 ml reaction volume containing 5 x 10^{-5} M xanthine, 52 µg/ml xanthine oxidase, and 10^{-5} M cytochrome c in 0.05 M potassium phosphate buffer with 10^{-4} M EDTA, pH 7.8. Statistically significant inhibition (p ≤ 0.05) of the reduction of cytochrome c occurred at concentrations of 10 mM (4%), 20 mM (10%), 38 mM (15%), and 107 mM (34%) taurine. Taurine at 5 mM demonstrated no significant (p > 0.05) effect on cytochrome c reduction.
Figure 14. The effect of hypotaurine on ferricytochrome c reduction by superoxide radicals. Experimental conditions were as described under "Materials and Methods". Each data point represents the mean ± SEM with n = 9. Means with a "**" above them were found to be significantly different (p ≤ 0.05) when compared to the control. Hypotaurine was added to a 1 ml reaction volume containing 5×10⁻⁵M xanthine, 52 µg/ml xanthine oxidase, and 10⁻⁵ M cytochrome c in 0.05 M potassium phosphate buffer with 10⁻⁴ M EDTA, pH 7.8. Statistically significant, but very modest inhibition (p ≤ 0.05) of the reduction of cytochrome c occurred at concentrations of 6 mM (4%), 8 mM (2%), and 10 mM (6%) hypotaurine. Hypotaurine at 2 mM and 4 mM demonstrated no significant effect (p > 0.05) on cytochrome c reduction.
E. Spin Trapping Studies

The hydroxyl radicals generated in a Fenton-type system (using Fe\(^{3+}\) + Ascorbate + \(\text{H}_2\text{O}_2\) ) yield spin adducts with DMPO (98). Figure 15 shows a well characterized 1:2:2:1 pattern of the DMPO-OH adduct with \(A_N = 14.92\)g. The DMPO-OH spectrum was generated when 2 mM \(\text{H}_2\text{O}_2\) was added to 0.1 mM ascorbate and 60 \(\mu\)M EDTA-chelated (50 \(\mu\)M) FeCl\(_3\) in the presence of 52 mM DMPO in 100 mM NaCl/25 mM NaHCO\(_3\) buffer, pH 7.0. The EPR signal of DMPO-OH was stable for 1 hour.

Addition of thiourea, a known ·OH scavenger, inhibited the intensity of the EPR signal in a dose-dependent manner. Thus, 6, 12, and 18 mM thiourea inhibited the signal intensity by 5, 24 and 42%, respectively (Figure 15).

The effect of taurine on the DMPO-OH spectrum was determined using the same EPR parameters (Figure 16). Taurine inhibited the signal intensity at all concentrations except the lowest, 5 mM, concentration. Thus taurine at 50, 75, 100, 200 and 300 mM inhibited the DMPO-OH spectrum by 5, 9, 22, 32 and 45%, respectively.

Hypotaurine also had an inhibitory effect on the DMPO-OH spectrum intensity at the three concentrations tested (Figure 17). At a concentration of 10 mM, hypotaurine inhibited the signal intensity by 9%. Hypotaurine added at 20 mM and 30 mM inhibited the signal intensity by 40% and 42%, respectively.

DMPO also forms a spin adduct with the superoxide radical, DMPO-OOH, which was characterized using a xanthine/xanthine oxidase system to produce superoxide radicals.
Superoxide dismutase (SOD) was utilized as a control inhibitor in this experiment. SOD (6.45 units/ milliliter of activity) virtually abolished the signal (Figure 18).

The addition of taurine inhibited the DMPO-OOH spectrum by a very small increment and only at the highest concentrations tested (Figure 19). At 5, 50, and 70 mM, taurine had no apparent effect on the signal of the adduct. The spectrum was inhibited by 11 and 34% by 148 and 193 mM taurine, respectively. Hypotaurine reduced the spectrum intensity by a maximum of 18% at concentrations of 13 mM and 19 mM (Figure 20). At 6 mM, hypotaurine inhibited the superoxide signal by 11%.
Figure 15. Effect of the hydroxyl radical scavenger, thiourea, on the DMPO-OH electron paramagnetic resonance spectrum. The DMPO-OH spectrum (line 1) was generated as described under "Materials and Methods." Each 0.1 ml reaction mixture contained 50 μM FeCl₃, 60 μM EDTA, 52 mM DMPO, 0.1 mM ascorbate, and 2 mM H₂O₂. Thiourea was added at 6 (line 2), 12 (line 3) and 18 mM (line 4) to the reaction mixture. The addition of H₂O₂ was used to start the reaction. Thiourea inhibited the DMPO-OH spectrum by 5% at 6 mM, by 24% at 12 mM, and by 42% at 18 mM. Line 5 is the EPR spectrum for DMPO alone.
Figure 16. Effect of taurine on the DMPO-OH electron paramagnetic resonance spectrum. The DMPO-OH spectrum (line 1) was generated as described under "Materials and Methods." Each 0.1 ml reaction mixture contained 50 μM FeCl₃, 60 μM EDTA, 52 mM DMPO, 0.1 mM ascorbate, and 2 mM H₂O₂ in 100 mM NaCl/25 mM NaHCO₃ buffer, pH 7.0. Taurine was added at 5 (line 2), 50 (line 3), 75 (line 4), 100 (line 5), 200 (line 6), and 300 mM (line 7) to the reaction mixture. The addition of H₂O₂ was used to start the reaction. Taurine inhibited the DMPO-OH spectrum by 5% at 50 mM, by 9% at 75 mM, by 22% at 100 mM, by 32% at 200 mM, and by 45% at 300 mM. Taurine had no effect on the DMPO-OH spectrum at 5 mM.
Figure 17. Effect of hypotaurine on the DMPO-OH electron paramagnetic resonance spectrum. The DMPO-OH spectrum (line 1) was generated as described under "Materials and Methods." Each 0.1 ml reaction mixture contained 50 μM FeCl₃, 60 μM EDTA, 52 mM DMPO, 0.1 mM ascorbate, and 2 mM H₂O₂ in 100 mM NaCl/25 mM NaHCO₃ buffer, pH 7.0. Hypotaurine was added at 10 (line 2), 20 (line 3), and 30 mM (line 4) to the reaction mixture. The addition of H₂O₂ was used to start the reaction. Hypotaurine inhibited the DMPO-OH spectrum by 9% at 10 mM, by 40% at 20 mM, and by 42% at 30 mM.
Figure 18. Effect of superoxide dismutase on the DMPO-OOH electron paramagnetic resonance spectrum. The DMPO-OOH spectrum (line 1) was generated as described under "Materials and Methods." Each 0.155 ml reaction mixture contained 210 mM DMPO, 1.6 x 10^{-4} M xanthine, and 1 mg/ml xanthine oxidase in 0.05 M potassium phosphate/ 10^{-4} M EDTA buffer, pH 7.8, with 35% DMSO. Xanthine oxidase was used to start the reaction. SOD was added to the reaction prior to xanthine oxidase at a final activity of approximately 6.45 units per ml. This level of SOD activity was sufficient to virtually abolish the DMPO-OOH spectrum (line 2). The labels a., b., c., and d. designate the peaks that are representative of the EPR spectrum of the superoxide DMPO-OOH adduct.
Figure 19. Effect of taurine on the DMPO-OOH electron paramagnetic resonance spectrum. The DMPO-OOH spectrum was generated as described under "Materials and Methods." Each 0.155 ml reaction mixture contained 210 mM DMPO, 1.6 x 10^-4 M xanthine, and 1 mg/ml xanthine oxidase in 0.05 M potassium phosphate/10^-4 M EDTA buffer, pH 7.8 with, 35% DMSO. Xanthine oxidase was used to start the reaction. Taurine was added to the reaction prior to xanthine oxidase at 5 (line 2), 50 (line 3), 77 (line 4), 148 (line 5), and 193 mM (line 6). Taurine inhibited the DMPO-OOH spectrum at 148 and 193 mM by 11 and 34%, respectively. At lower concentrations, taurine had no effect on the DMPO-OOH spectrum (control = line 1). The labels a., b., c., and d. designate the peaks that are representative of the EPR spectrum of the superoxide DMPO-OOH adduct.
Figure 20. Effect of hypotaurine on the DMPO-OOH electron paramagnetic resonance spectrum. The DMPO-OOH spectrum was generated as described under "Materials and Methods." Each 0.155 ml reaction mixture contained 210 mM DMPO, 1.6 x 10^{-4} M xanthine, and 1 mg/ml xanthine oxidase in 0.05 M potassium phosphate/10^{-4} M EDTA buffer, pH 7.8, with 35% DMSO. Xanthine oxidase was used to start the reaction. Hypotaurine was added to the reaction prior to xanthine oxidase at 6, 13, and 19 mM (lines 2, 3, and 4, respectively). Hypotaurine inhibited the DMPO-OOH (control = line 1) spectrum at 19 mM by 5%. At lower concentrations, hypotaurine had no effect on the DMPO-OOH spectrum. The labels a., b., c., and d. designate the peaks that are representative of the EPR spectrum of the superoxide DMPO-OOH adduct.
F. *Ex vivo* Model of Ischemia-Reperfusion Injury

The three indices used to assess lung injury during reperfusion were percentage change in pulmonary artery pressure (pre-ischemic period versus post-ischemic ventilation period), percentage change in peak airway pressure (pre-ischemic period versus post-ischemic ventilation period), and wet-to-dry weight ratio (an index of edema formation). Groups of lungs (n=6) were treated as previously described and the mean percentage change values calculated for each group were compared to those of the other groups, as was the mean wet-to-dry weight ratio. For each lung, the pre-ischemic period served as its control.

A representative tracing from an uninjured control lung (Figure 21) has been included for clarity of presentation of subsequent data from the MacLab system using Chart® software (World Precision Instruments, New Haven, CT). The injured controls and taurine-treated lungs had similar tracings. All attempts at hypotaurine treatment were unsuccessful because the lungs were irreparably damaged by filling with fluid before adequate data collection could be achieved. Therefore, the hypotaurine treatment groups were aborted and no conclusive data was obtained. Additionally, 4 out of a total of 5 *ex vivo* preparations attempted in preliminary studies using 50 mM taurine were aborted due to the lungs filling with fluid.

The mean percentage change in pulmonary artery pressure (P_a) increased for all lungs in which there was a change. As shown in Table 1, the uninjured lungs were shown to have no change in P_a between the pre-ischemic and the post-ischemic ventilation period, whereas in the injured lungs, 5mM taurine and 10 mM taurine lungs demonstrated a mean increase in P_a of 38, 11.83, and 53.33%, respectively. Means
followed by the same letter were not statistically different at the $\alpha = 0.05$ significance level with a $p$ value of $p \leq 0.05$ considered to be significantly different.

The mean wet-to-dry weight ratios for the four lung groups are shown in Table 2. The Uninjured lungs were shown to have a mean wet-to-dry weight ratio of 6.1, whereas the Injured, 5 mM Taurine, and 10 mM Taurine lungs were shown to have mean wet-to-dry weight ratios of 10.25, 8.39, and 10.47, respectively. Means followed by the same letter were not statistically different at the $\alpha = 0.05$ significance level with a $p$ value of $p \leq 0.05$ considered to be significantly different.

The mean change in peak airway pressure ($P_{aw}$) was an increase in all lungs. As shown in Table 3, the Uninjured group was shown to have a mean $P_{aw}$ increase of 6.33, whereas the Injured, 5 mM Taurine, and 10 mM Taurine lungs were shown to have mean $P_{aw}$ increases of 15.33, 4.83, and 17.33, respectively. Means followed by the same letter were not statistically different at the $\alpha = 0.05$ significance level with a $p$ value of $p \leq 0.05$ considered to be significantly different.

Table 4 and 5 present a summary of the effects of reagents on the EPR spectra of DMPO-OH and DMPO-OOH, respectively. Tables 6 and 7 present a summary of data collected in the *in vitro* studies for taurine and hypotaurine, respectively.
Figure 21. Representative tracing from Chart© software for an uninjured control lung. Experimental conditions were as described in "Materials and Methods." After removal from the rat, lungs were perfused for a total of 100 minutes (Uninjured lungs) or perfused for 10 minutes followed by 60 minutes of ischemia followed by 30 minutes of reperfusion (Injured and Taurine-treated lungs). This figure is representative of the lung weight, pulmonary artery pressure, and peak airway pressure data collected for the Uninjured lungs.
### Table 1. Mean change in pulmonary artery pressure among the four treatment groups.

Experimental conditions were as described in "Materials and Methods." Pulmonary artery pressure (Pa) was observed among the groups during the initial 10 minutes of perfusion (pre-ischemic = 19.5 mmHg) and for the last 25 minutes of reperfusion (post-ischemic). A percentage change was calculated by comparing the mean of the pre-ischemic Pa values with the mean of the post-ischemic Pa values. A one-way analysis of variance (ANOVA) was performed to determine statistically significant differences between the lung groups (Uninjured, Injured, Taurine 5 mM, and Taurine 10 mM). Subsequently, Fisher's Least Significant Difference (LSD) multiple comparison test was performed to determine which group means differed from the others at a significance level of $\alpha = 0.05$. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Mean Pa</th>
<th>% Increase *</th>
<th>Minimum</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine 10 mM</td>
<td>6</td>
<td>53.3a</td>
<td>5 - 118%</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>Injured</td>
<td>6</td>
<td>38.0ab</td>
<td>15 - 77%</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>Taurine 5 mM</td>
<td>6</td>
<td>11.8bc</td>
<td>0 - 32%</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>Uninjured</td>
<td>6</td>
<td>0c</td>
<td>0%</td>
<td>11.1</td>
<td></td>
</tr>
</tbody>
</table>

* Means followed by the same letter are not significantly different at the 0.05 level (comparisonwise), using Fisher's LSD (99).

n = sample size

SEM = Standard error of the mean, assuming equivalence of variance
<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Mean Wet/Dry</th>
<th>Wet/Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine 10 mM</td>
<td>6</td>
<td>10.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0 - 13.8 0.79</td>
</tr>
<tr>
<td>Injured</td>
<td>6</td>
<td>10.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.3 - 12.1 0.79</td>
</tr>
<tr>
<td>Taurine 5 mM</td>
<td>6</td>
<td>8.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.6 - 10.3 0.79</td>
</tr>
<tr>
<td>Uninjured</td>
<td>6</td>
<td>6.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.2 - 7.1 0.79</td>
</tr>
</tbody>
</table>

* Means followed by the same letter are not significantly different at the 0.05 level (comparisonwise), using Fisher's LSD (99).

n = sample size
SEM = Standard error of the mean, assuming equivalence of variance

Table 2. Wet-to-dry weight ratios of the four treatment groups. Experimental conditions were as described in "Materials and Methods." Immediately following the conclusion of reperfusion, the lungs were dissected from the heart, trachea and large bronchus structures. The lungs were weighed (wet weight) and placed in a convection oven at 120°C for 72 hours (until no further weight loss was detected). At that time, a dry weight was determined and the wet-to-dry weight ratio calculated. A one-way analysis of variance (ANOVA) was performed to determine statistically significant differences between the animal groups (Uninjured, Injured, Taurine 5 mM, and Taurine 10 mM). Subsequently, Fisher's LSD multiple comparison test was performed to determine which groups differed from the others at a significance level of \( \alpha = 0.05 \).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Mean P&lt;sub&gt;aw&lt;/sub&gt;</th>
<th>% Increase</th>
<th>Minimum</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine 10 mM</td>
<td>6</td>
<td>17.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 - 28%</td>
<td>3.6</td>
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<tr>
<td>Injured</td>
<td>6</td>
<td>15.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0 - 33%</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Uninjured</td>
<td>6</td>
<td>6.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4 - 10%</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Taurine 5 mM</td>
<td>6</td>
<td>4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 - 16%</td>
<td>3.6</td>
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</tbody>
</table>

* Means followed by the same letter are not significantly different at the 0.05 level (comparisonwise), using Fisher's LSD (99).

n = sample size
SEM = Standard error of the mean, assuming equivalence of variance

Table 3. Mean change in peak airway pressure among the four treatment groups. Experimental conditions were as described in "Materials and Methods." Peak airway pressure (P<sub>aw</sub>) was observed among the groups during the initial 10 minutes of perfusion (pre-ischemic = 4.4 mmHg) and for the last 25 minutes of reperfusion (post-ischemic). A percentage change was calculated by comparing the mean of the pre-ischemic P<sub>aw</sub> values with the mean of the post-ischemic P<sub>aw</sub> values. A one-way analysis of variance (ANOVA) was performed to determine statistically significant differences between the animal groups (Uninjured, Injured, Taurine 5 mM, and Taurine 10 mM). Subsequently, Fisher's LSD multiple comparison test was performed to determine which groups differed from the others at a significance level of \( \alpha = 0.05 \).
Table 4. DMPO-OH SPECTRUM - Effect of reagents tested

<table>
<thead>
<tr>
<th>Reagent</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mM Thiourea</td>
<td>5%</td>
</tr>
<tr>
<td>12 mM Thiourea</td>
<td>24%</td>
</tr>
<tr>
<td>18 mM Thiourea</td>
<td>42%</td>
</tr>
<tr>
<td>5 mM Taurine</td>
<td>no inhibition</td>
</tr>
<tr>
<td>50 mM Taurine</td>
<td>5%</td>
</tr>
<tr>
<td>75 mM Taurine</td>
<td>9%</td>
</tr>
<tr>
<td>100 mM Taurine</td>
<td>22%</td>
</tr>
<tr>
<td>200 mM Taurine</td>
<td>32%</td>
</tr>
<tr>
<td>300 mM Taurine</td>
<td>45%</td>
</tr>
<tr>
<td>10 mM Hypotaurine</td>
<td>9%</td>
</tr>
<tr>
<td>20 mM Hypotaurine</td>
<td>40%</td>
</tr>
<tr>
<td>30 mM Hypotaurine</td>
<td>42%</td>
</tr>
</tbody>
</table>

Table 5. DMPO-OOH SPECTRUM - Effect of reagents tested

<table>
<thead>
<tr>
<th>Reagent</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.45 units of activity/ml SOD</td>
<td>100%</td>
</tr>
<tr>
<td>5 mM Taurine</td>
<td>no inhibition</td>
</tr>
<tr>
<td>50 mM Taurine</td>
<td>no inhibition</td>
</tr>
<tr>
<td>77 mM Taurine</td>
<td>no inhibition</td>
</tr>
<tr>
<td>148 mM Taurine</td>
<td>11%</td>
</tr>
<tr>
<td>193 mM Taurine</td>
<td>34%</td>
</tr>
<tr>
<td>6 mM Hypotaurine</td>
<td>no inhibition</td>
</tr>
<tr>
<td>13 mM Hypotaurine</td>
<td>no inhibition</td>
</tr>
<tr>
<td>19 mM Hypotaurine</td>
<td>5%</td>
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</table>
**Table 6. SUMMARY TABLE TAURINE**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive Effect**</th>
<th>Negative Effect@</th>
<th>No Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation of Macrophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation of $H_2O_2$ production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by macrophages</td>
<td>13 -104 mM</td>
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<tr>
<td>Microsomal lipid peroxidation</td>
<td></td>
<td></td>
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<tr>
<td>Deoxyribose degradation</td>
<td>5 - 100 mM</td>
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<tr>
<td>Ferricytochrome c reduction</td>
<td>10 - 107 mM</td>
<td>5 mM</td>
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</tr>
<tr>
<td>Spin Trapping</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPO-OH signal</td>
<td>50 - 300 mM</td>
<td>5 mM</td>
<td></td>
</tr>
<tr>
<td>DMPO-OOH signal</td>
<td>148 - 193 mM</td>
<td>5 - 77 mM</td>
<td></td>
</tr>
</tbody>
</table>

** Positive effect = an increase or augmentation of the activity or damaging effect.

@ Negative effect = a decrease or inhibition of the activity or damaging effect.

◆ Taurine had no effect at any concentration tested (5, 20, 40, 120, and 160 mM)

◆◆ Taurine had no effect at any concentration tested (5, 10, 20, 40, and 120 mM)
### Table 7. SUMMARY TABLE HYPOTAURINE

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive Effect**</th>
<th>Negative Effect @</th>
<th>No Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation of Macrophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by macrophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation of H₂O₂ production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by macrophages</td>
<td>3.2 mM</td>
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<td>Microsomal lipid peroxidation</td>
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<tr>
<td>Deoxyribose degradation</td>
<td>2 - 10 mM</td>
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</tr>
<tr>
<td>Ferricytochrome c reduction</td>
<td>6 - 10 mM</td>
<td>2 - 4 mM</td>
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<tr>
<td>Spin Trapping</td>
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<tr>
<td>DMPO-OH signal</td>
<td>10 - 30 mM</td>
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<tr>
<td>DMPO-OOH signal</td>
<td>19 mM</td>
<td>6, 13 mM</td>
<td></td>
</tr>
</tbody>
</table>

** Positive effect = an increase or augmentation of the activity or damaging effect.

@ Negative effect = a decrease or inhibition of the activity or damaging effect.

♦ Hypotaurine had no effect at any concentration tested (0.1, 0.4, 0.8, 2.4, and 3.2 mM)

♦♦ Hypotaurine had no effect at any concentration tested (2, 4, 6, 8, and 10 mM)
Chapter 4
DISCUSSION

Taurine and its metabolite, hypotaurine, have been characterized as having antioxidant properties by many investigators based upon very strong, but indirect, evidence. Based upon results in the present study, hypotaurine and taurine may be definitively classified as antioxidants. Direct evidence as well as additional indirect evidence was collected in support of the ability of both of these sulfur-containing amino acids to interact with and scavenge reactive species of oxygen. Hypotaurine and taurine possess different scavenging abilities within different experimental systems and with respect to the different oxygen intermediates. It is also possible that they possess the capacity to perform multiple functions, by different mechanisms, in the cell in addition to their antioxidant function.

A. Effect of Taurine and Hypotaurine on H₂O₂ Production by Macrophages

Hypotaurine demonstrated no significant inhibition of H₂O₂ levels in PMA-stimulated macrophages under the experimental conditions of the study. It appears, therefore, that hypotaurine does not scavenge this particular reactive species of oxygen.

Taurine produced somewhat paradoxical results in that it demonstrated no significant stimulation of macrophages when added to the cells in the absence of PMA, however in the presence of PMA, 13, 26, 78, and 104 mM taurine augmented H₂O₂ production significantly by 9, 13, 28, and 43% above that present in the positive control (Figure 5). PMA is a soluble pharmacological agent that is capable of stimulating oxidative metabolism associated with phagocytosis (87). The stimulation of macrophages with
PMA induces an "oxidative metabolic burst" within the phagocytic cell by activating protein kinase C which, in turn, activates other enzymes in the cell. Ultimately, superoxide anion is generated via a one-electron reduction of molecular oxygen by NADPH-oxidase (100-102). The superoxide anions so generated dismutate either spontaneously or via the action of SOD to form $\text{H}_2\text{O}_2$, which then reacts with halide ions via the action of myeloperoxidase, to generate hypochlorous acid and chloramines inside the phagosome (103).

In the presence of taurine the chloramine, monochlorotaurine, is formed by the myeloperoxidase system (104). It has been shown that monochlorotaurine is more stable than other monochloramines because it is the chlorinated form of a beta-amino acid rather than that of an alpha-amino acid. Chlorinated alpha-amino acids spontaneously deaminate, decarboxylate, and dechlorinate to form reactive aldehydes within 5 minutes of their formation, whereas the chlorinated beta-amino acid, monochlorotaurine, has a half-life of approximately 2.5 days (61). Other properties of monochlorotaurine include its ability to inhibit the myeloperoxidase reaction (105,106) and, although monochlorotaurine is an oxidant, it reacts with relatively fewer organic substances to become reduced (61).

It is possible that the resultant increase of $\text{H}_2\text{O}_2$ production by macrophages exposed to both PMA and taurine in the experimental system used in this study was due to the ability of taurine to scavenge the hypochlorous acid produced by myeloperoxidase, thereby forming the stable byproduct, monochlorotaurine. As the levels of monochlorotaurine accumulated over the 45 minute incubation time, it is possible that
This byproduct exerted an inhibitory effect on the myeloperoxidase reaction, thereby allowing the levels of $\text{H}_2\text{O}_2$ to increase above that of cells stimulated with PMA alone.

Further evidence which supports this theory was found by Weiss and co-workers using the agents azide and cyanide. The use of either cyanide or azide effectively masked the cytotoxic effects of myeloperoxidase and at the same time allowed the damaging effects of $\text{H}_2\text{O}_2$ to be demonstrated. These investigators found that, under conditions where myeloperoxidase activity was effectively masked and cytotoxicity could be almost completely attributable to $\text{H}_2\text{O}_2$, normal neutrophils exhibited markedly stimulated cytotoxicity toward endothelial cells (107). Weiss et al. concluded that the increased cytotoxicity reflected an increased availability of $\text{H}_2\text{O}_2$ secondary to the inhibition of catalase or myeloperoxidase. Weiss et al. further characterized the mechanism of endothelial cell cytolysis by testing neutrophils from patients with myeloperoxidase-deficient lysosomes. It has also been shown that myeloperoxidase-deficient cells generate more superoxide, hydrogen peroxide and perhaps hydroxyl radicals than normal neutrophils but appear to damage endothelial cells by $\text{H}_2\text{O}_2$ alone (107-109).

It is possible that taurine had its stimulatory effect on RAW 264.7 macrophage $\text{H}_2\text{O}_2$ production indirectly by acting as a scavenger of hypochlorous acid. The byproduct of the scavenging process, monochlorotaurine, may have inhibited the myeloperoxidase system which lead to an increased production of reactive oxygen species, mainly measured by increased hydrogen peroxide. Therefore, while taurine does not appear to scavenge hydrogen peroxide, the biological importance of its ability to scavenge the very powerful oxidant, hypochlorous acid, is once again highlighted.
B. Effect of Taurine and Hypotaurine on Enzymatically-Induced Microsomal Lipid Peroxidation

Lipid peroxidation is believed to play an important role in the toxicity of various xenobiotics and to contribute to or initiate cell damage via membrane degradation. The process of lipid peroxidation can be initiated by any chemical species which possesses enough reactivity to abstract a hydrogen atom from a polyunsaturated fatty acid methylene group. In aerobic cells, the most likely initiating species are oxy-radicals (or complexes of oxy-radicals with ADP-iron) generated by normal cellular processes such as the detoxification of xenobiotics or endogenous oxidation reactions.

Lipid peroxidation proceeds as a two step process: initiation reactions followed by propagation reactions. There is still some question about what specific reactive species of oxygen initiates the process, however, it has been proposed by some investigators that initiation can occur via the action of singlet oxygen, hydroxyl radicals, and via formation of an ADP-perferryl ion either through the production of superoxide by xanthine oxidase (110) or by NADPH-cytochrome P₄₅₀ reductase (111) or a combination of the two systems working in concert. Initiation of lipid peroxidation leads ultimately to the formation of lipid hydroperoxides that are not particularly stable and are catalyzed to decompose by their interaction with trace amounts of transition metal ions to form other lipid radicals (112). This decomposition, catalyzed by trace transition metal ions, is the propagation phase of the lipid peroxidation process.

One of the end products of lipid peroxidation is MDA which reacts under acidic conditions with thiobarbituric acid to produce a colored end product. This end product reaction has been widely utilized and has been deemed reliable to assess the extent of
lipid peroxidation in vitro (113). This method was utilized in the present study to
detect NADPH-dependent lipid peroxidation in bovine lung microsomes and to assess
the effect of several potential antioxidants within this in vitro system.

Mannitol has been shown to react with and quench the hydroxyl radical (92). Thiourea
has also been demonstrated to directly scavenge the hydroxyl radical (95). However,
the hydroxyl radical is not believed to be the major species responsible for initiation or
propagation of lipid peroxidation, even though its reactive nature renders it capable of
doing so. The reason for this is because investigators have shown that NADPH-
dependent lipid peroxidation is not significantly inhibited by the addition of hydroxyl
radical scavengers such as benzoate (114). It has also been postulated by some that the
hydroxyl radical is so extremely reactive that it would most likely be scavenged by
biological molecules other than unsaturated lipids of the cell membrane (115). The fact
that mannitol only inhibited 4% and thiourea only inhibited 16% (each at 10 mM) of
the lipid peroxidation present in the control is consistent with the theory that the
hydroxyl radical is not the major initiating or propagating agent of NADPH-dependent
lipid peroxidation.

The use of manganese salts as antioxidant drugs in equine clinical medicine has been
proposed (94) because such salts possess the ability to scavenge superoxide anion
radicals (93). In the present study, 10 mM manganese chloride was able to inhibit 22%
of the lipid peroxidation found in the control. Inhibition of 22% was less than expected
considering previous findings implicating the superoxide radical, formed via the action
of the enzyme xanthine oxidase (110), as a key player in the formation of the ADP-
perferryl ion and thus in the initiation of lipid peroxidation. In the same study, Svingen
et al. have shown that the ADP-perferryl ion-catalyzed initiation of NADPH-dependent lipid peroxidation in rat hepatic microsomes was entirely inhibited by superoxide dismutase indicating its dependence upon the superoxide radical. On the other hand, Pederson and Aust proposed that the ADP-perferryl ion was formed in rat liver microsomes by the action of NADPH-cytochrome P₄₅₀ reductase in the presence of oxygen (111). It has been proposed that the xanthine oxidase system and the P₄₅₀ reductase system each contribute to the initiation of lipid peroxidation via the formation of the ADP-perferryl ion.

The lung has been shown to possess the ability to convert xanthine dehydrogenase to xanthine oxidase in response to an ischemic event, however, its rate of conversion is much slower in lung than other tissues such as heart, brain, and intestine (116). The lung has also been shown to be well endowed with P₄₅₀ cytochromes (32). The result of the present study, with respect to the action of manganese chloride in the bovine lung microsome system, is consistent with a mechanism of initiation of lipid peroxidation involving the formation of the ADP-perferryl ion occurring mainly by the action of the NADPH-cytochrome P₄₅₀ reductase rather than via the xanthine oxidase system, which may play a smaller role in lung than other tissues. In this case, one would not expect the formation of the ADP-perferryl ion by NADPH-cytochrome P₄₅₀ reductase, and therefore the initiation of lipid peroxidation by the action of a by-product of this enzymatic reaction, to be inhibited by SOD or other superoxide scavenging agents such as manganese chloride.

Diethylenetriamine pentaacetic acid (DETAPAC) has previously been used to inhibit metal-catalyzed lipid peroxidation by Thomas and associates (117). Both the initiation
and propagation of lipid peroxidation are believed to be catalyzed by transition metal ions thus explaining the ability of DETAPAC to inhibit 87% of the lipid peroxidation present in the positive control (Figure 7).

Conflicting results have been obtained in experiments performed to determine the effect of taurine in lipid peroxidation systems (Table 8). Taurine has been shown to protect against lipid peroxidation initiated in various systems by various compounds, however, it demonstrated no significant effect (p > 0.05) in the present study. The lack of effect in the present study is consistent with similar results found by other investigators listed in Table 8. The reason for this may have to do with the complexity of the process of lipid peroxidation and the many in vitro mechanisms which may be utilized to study it. In addition, the cell membrane itself is a very complex environment which hinders our ability to have a clear understanding of lipid peroxidation in vivo.

In many of the investigations in which taurine demonstrated no effect on lipid peroxidation or demonstrated a protective effect via some other mechanism, the authors propose a mechanism whereby taurine exerts its effect by modulation of calcium influx. Nakamura and associates found that taurine suppressed erythrocyte hemolysis by the oxygen-radical generator 2,2'-azobis-(2-amidinopropane)dihydrochloride (AAPH), however, when they further investigated the relationship of suppression of hemolysis by taurine to its inhibition of lipid peroxidation, taurine failed to inhibit lipid peroxidation in a system using liposomes prepared with egg yolk lecithin (118). The same investigators subjected erythrocytes to hemolysis by hyposmotic solutions and, in this case, taurine was effective at inhibiting hemolysis. From this result, they concluded
that taurine did not have an antioxidant effect but, instead, interacted with the membrane in some way which protected it from the damaging effects of AAPH.

Nakashima et al. have demonstrated that taurine protected isolated rat hepatocytes during calcium and oxygen paradoxes. Taurine inhibited oxygenation-induced lipid peroxidation and prevented cell death in a calcium-containing medium, but not in a calcium-free medium, leading the authors to conclude that taurine protected by a mechanism involving calcium influx (85). Other studies have implicated taurine's ability to modulate calcium influx especially in the heart (68,69,79-86). The data spin a confusing web which still requires further study to determine and separate, if possible, the antioxidant effects of taurine from its involvement in cellular calcium homeostasis. The fact that we demonstrated no effect of taurine in the NADPH-dependent lipid peroxidation system is consistent with other studies which found taurine to have no effect on lipid peroxidation but inconsistent with others that found taurine to be significantly inhibitory of lipid peroxidation (see Table 8). In those studies where taurine provided protection, it is possible that taurine exerts its membrane protective properties by a complex combination of interactions which utilize all of its individually confirmed properties as an antioxidant, a membrane stabilizer, and a modulator of calcium homeostasis.

Our results in the NADPH-dependent lipid peroxidation system indicate that neither taurine (5-120 mM) nor hypotaurine (2-10 mM) possess as strong an ability to inhibit the portion of lipid peroxidation caused by the hydroxyl radical as 10 mM thiourea or mannitol. This result is consistent with evidence suggesting a minor role for the hydroxyl radical in NADPH-dependent lipid peroxidation. The results also indicate
that taurine and its precursor, hypotaurine, failed to demonstrate inhibition of lipid peroxidation equal to that of the superoxide scavenger, 10 mM manganese chloride, and that taurine and hypotaurine do not interact with iron in such a way that is inhibitory to iron-dependent lipid peroxidation.
Table 8. Taurine and Hypotaurine: Effects in various models of lipid peroxidation

<table>
<thead>
<tr>
<th>Lipid Peroxidation Model</th>
<th>Effect</th>
<th>Ref. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous lipid peroxidation</td>
<td>• 0.5 mM hypotaurine: inhibited by 53%</td>
<td>72</td>
</tr>
<tr>
<td>• Induced by exposure of spermatozoa to O₂ during incubation (believed to be mediated by O₂⁻)</td>
<td>• 0.5 mM taurine: inhibited by 29%</td>
<td></td>
</tr>
<tr>
<td>Hydroperoxide-initiated chemiluminescence</td>
<td>• 5 gram intravenous taurine pretreatment: decreased oxidative stress ratio by 50%</td>
<td>75</td>
</tr>
<tr>
<td>• Detected in biopsy samples from patients undergoing bypass surgery</td>
<td>• 2.5% taurine dietary supplement (6-day pretreatment) did not significantly decrease MDA formation</td>
<td>119</td>
</tr>
<tr>
<td>Malondialdehyde (MDA) formation in rat lungs treated with Amiodarone</td>
<td>• 1 mM taurine added to the reaction medium inhibited lipid peroxidation</td>
<td>120</td>
</tr>
<tr>
<td>MDA formation after exposure of liposomes to the myeloperoxidase-H₂O₂-Cl system of activated rat neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-induced lipid peroxidation in cultured rat mesangial cells</td>
<td>• 500 µM taurine prevented the glucose-induced increase in lipid peroxidation</td>
<td>121</td>
</tr>
<tr>
<td>MDA levels in perfused heart muscle</td>
<td>• 10⁻³ M and 10⁻² M taurine: 1. administered before ischemia-reperfusion: increased MDA levels</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>2. administered after ischemia-reperfusion: decreased MDA levels</td>
<td></td>
</tr>
<tr>
<td>Erythrocyte hemolysis</td>
<td>• taurine suppressed erythrocyte hemolysis but the effect was deemed to be osmotic rather than antioxidant</td>
<td>118</td>
</tr>
<tr>
<td>• Induced by 2,2’Azobis(2amidinopropane) dihydrochloride, an oxygen radical generator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugar Cataractogenesis</td>
<td>• 0.2 mM taurine was shown to protect the lens against development of sugar-induced cataracts</td>
<td>123</td>
</tr>
<tr>
<td>• MDA levels used as measurement of lipid peroxidation</td>
<td>• taurine was inversely related to MDA production, suggesting an antioxidant effect</td>
<td></td>
</tr>
<tr>
<td>Bleomycin-induced interstitial pulmonary fibrosis</td>
<td>• 1% taurine in drinking water plus 250 mg/kg niacin via intraperitoneal (i.p.) injection daily: decreased bleomycin-induced increase in MDA formation</td>
<td>124</td>
</tr>
<tr>
<td>• associated with increased lipid peroxidation (and other parameters) measured by MDA formation</td>
<td>• up to 50 mM taurine failed to affect lipid peroxidation</td>
<td>125</td>
</tr>
<tr>
<td>Cadmium chloride- or L-ascorbic acid-induced lipid peroxidation in membrane fractions and isolated bovine retinal cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
C. Effects of Taurine and Hypotaurine on Hydroxyl Radical Mediated-Deoxyribose Degradation

In our study the deoxyribose assay was utilized to determine the ability of taurine and hypotaurine to react with and scavenge the hydroxyl radical. Hypotaurine demonstrated dosedependent inhibition of the degradation of deoxyribose and when plotted as described by Halliwell et al. the hypotaurine data conformed to a linear competition plot. These results are consistent with evidence reported by Fellman and Roth in 1985 and by Alvarez and Storey in 1983. Fellman and Roth reported that hypotaurine exhibited a profound hydroxyl free radical quenching effect when added to a xanthine/xanthine oxidase system in the presence of EDTA-Fe^{2+} (74). Hypotaurine also demonstrated a protective effect when added to a rabbit sperm, spontaneous lipid peroxidation system (70). Green and associates demonstrated that hypotaurine could effectively compete with DMPO for hydroxyl radicals (126). Hypotaurine appears to possess significant antioxidant properties with respect to its ability to scavenge the hydroxyl radical.

Taurine (100 mM) was able to inhibit up to 79% of the deoxyribose degradation present in the control, however, when a competition plot was attempted the inhibition proved to be non-linear and, therefore, not dosage dependent. An explanation for this may be provided by examining the non-linear competition plot obtained by Moorhouse et al. for the hydroxyl radical scavenger, thiourea (127). In studies done by Moorhouse et al. thiourea showed marked deviations from linearity, suggesting to the investigators that thiourea was not acting solely as a scavenger of the hydroxyl radical. While it is generally accepted that thiourea reacts with the hydroxyl radical and reacts with a relatively high second order rate constant (greater than that of mannitol and
ethanol), the results of the Moorhouse study suggest that thiourea is having effects in addition to radical scavenging. It appears that taurine may be similar to thiourea in that it appears to possess the ability to scavenge hydroxyl radicals, but, at the same time, may be having some other effect on deoxyribose degradation.

D. Effect of Taurine and Hypotaurine on Superoxide Radical-Mediated Ferricytochrome c Reduction

Taurine and hypotaurine demonstrated limited ability to inhibit superoxide-dependent reduction of ferricytochrome c. The maximum inhibition (34%) was achieved when 107 mM taurine was added to the reaction mixture. Hypotaurine was able to achieve only 6% inhibition at 10 mM. It appears that hypotaurine is a relatively poor scavenger of superoxide and that taurine only possesses scavenging ability at high concentrations.

E. Spin Trapping Studies

The data obtained for hypotaurine and taurine in spin trapping experiments (summarized in Table 4 and Table 5) were consistent with data obtained in the deoxyribose degradation (Figs. 10-12) and ferricytochrome c reduction (Figs. 13-14) experiments. When taurine was added to the Fenton-type system used to generate hydroxyl radical formation in the presence of the spin trap, DMPO, taurine demonstrated a maximum of 45% inhibition of the DMPO-OH spectrum at a 300 mM concentration. This result is consistent with the ability of taurine to inhibit 79% of the deoxyribose degradation if we consider that, in both cases, taurine was competing with another substance for the hydroxyl radical. In the deoxyribose experiments, 100 mM taurine was competing with 2.8 mM deoxyribose for the hydroxyl radicals produced by
the Fenton system to achieve 79% inhibition, whereas, in the spin trapping experiments 100 mM taurine was competing with 52 mM DMPO to achieve 22% inhibition. The net result was that there was 18.6 times more competitive substance present with which taurine had to compete in the spin trapping experiments, which resulted in 4 times lower inhibition than the same concentration of taurine in the deoxyribose system. Even so, these results indicate that high concentrations of taurine are required to demonstrate a direct hydroxyl radical scavenging effect.

Hypotaurine demonstrated a maximum of 42% inhibition of the DMPO-OH signal at a concentration of 30 mM and displayed 9% inhibition at 10 mM. Again this result is in agreement with the results of the deoxyribose assay where 10 mM hypotaurine inhibited 94% of the deoxyribose degradation present in the control if we consider the concentrations of competing reagents. The 10 mM hypotaurine was competing with 52 mM DMPO in the spin trapping experiments versus 2.8 mM deoxyribose in the deoxyribose experiments. The net effect was that competition with 18.6 times more competitive reagent resulted in 10 times lower inhibition by the same concentration of hypotaurine. This result may indicate that hypotaurine was present in too limited a concentration in the spin trapping reaction to compete as effectively as it did in the deoxyribose experiments where hypotaurine was in excess of its competitor in all but one instance. The results of the spin trapping studies do indicate that hypotaurine does indeed scavenge hydroxyl radicals in this very simple, chemical assay.

The DMPO-OOH spectrum was generated using a xanthine/xanthine oxidase system. The spectrum was completely inhibited by approximately 6.45 units/ml of SOD (Figure 18). Taurine demonstrated a maximum inhibition of 34% at a concentration of 193
mM (Figure 19) which is comparable to the result obtained in the ferricytochrome c experiments where 107 mM taurine inhibited the rate of ferricytochrome c reduction by 34% (Figure 13). In the spin trapping studies, taurine and hypotaurine were competing with 210 mM DMPO for the superoxide radicals formed by the xanthine/xanthine oxidase system compared to competition with 0.01 mM cytochrome c in the ferricytochrome c experiments. This amount of DMPO was necessary to obtain a strong, stable DMPO-OOH signal. The high concentration of DMPO may explain why it took 1.8 times more taurine in the spin trapping studies to achieve the same amount of inhibition demonstrated in the ferricytochrome c studies. These results indicate that taurine has the ability to scavenge superoxide, however, relatively high concentrations are required.

Hypotaurine at a concentration of 19 mM was able to inhibit the DMPO-OOH spectrum by only 5% (Figure 20) and was ineffective at demonstrating any inhibition at lower concentrations. This corresponds to the limited ability of hypotaurine to protect ferricytochrome c from reduction in earlier experiments thus leading to the conclusion that hypotaurine is a poor scavenger of superoxide anion radical.

**F. Ex vivo Model of Ischemia-Reperfusion Injury**

With respect to P_{aw}, the four experimental groups demonstrated no significant difference in percentage change values, indicating that no detectable airway damage occurred during the ischemia-reperfusion event in any of the isolated lung groups (Table 3).
These results demonstrated that the addition of taurine (5 mM and 10 mM) to the perfusion circuit was unable to offer significant protection to isolated rat lungs exposed to 60 minutes of ischemia followed by 30 minutes of reperfusion. This was determined by examining the effect of the addition of taurine on peak airway pressure ($P_{aw}$), pulmonary artery pressure ($P_a$) and wet-to-dry weight ratios. The $P_a$ remained stable over the 100 minutes of perfusion in the control lungs not subjected to ischemia (Uninjured). Lungs subjected to ischemia were allowed to reach a stable $P_a$ within 10 minutes after the onset of reperfusion. The $P_a$ was found to be significantly higher after the onset of reperfusion when compared to both the same lung during the pre-ischemic period and the time-matched uninjured control lung. The addition of taurine at 5 mM and 10 mM was unable to reduce significantly the $P_a$ of lungs exposed to ischemia followed by reperfusion compared to injured controls.

Lung weight remained stable in uninjured control lungs during 100 minutes of perfusion. Injured control lungs experienced a gradual increase in weight over the 30 minute period of reperfusion as measured by the linear force transducer attached to the MacLab-4 system via the transbridge. Wet-to-dry weight ratios served as a measure of pulmonary edema formation. The injured control lungs were found to have significantly higher wet-to-dry weight ratios than the uninjured controls (Table 2). The addition of 5 mM and 10 mM taurine was unable to provide significant reduction of the wet-to-dry weight ratios as compared to the injured controls.

In experiments where hypotaurine (2 and 4 mM) was added to the perfusion buffer, the isolated perfused rat lungs were unable to tolerate the procedure. In each isolated lung preparation exposed to 2 or 4 mM hypotaurine, the lungs either filled with fluid within
5 minutes of the initiation of perfusion or filled within 5 minutes of reperfusion following ischemia. No information could be obtained about toxicity studies done with hypotaurine. Hypotaurine is endogenous to lung tissue as is taurine (55). The ratio of taurine to hypotaurine in the cytosol of neutrophils has been reported to be 50:1 by Green et al. (126). If one assumes that the 50:1 ratio of taurine to hypotaurine in neutrophils is similar to that in other tissues, then the normal endogenous amount of hypotaurine in the rat lung would be approximately 0.1 mM. In that case, in these studies 20 to 40 times the endogenous amount of hypotaurine was added which, in theory, could be toxic to the lung and, by an undetermined mechanism, cause the rapid onset of edema. The addition of hypotaurine did not alter the pH of the solution, which remained constant at 7.4. Hypotaurine has been found to be a zwitterion at pH 7.4 and therefore would possess no charge. Additionally, 4 out of 5 ex vivo preparations attempted in preliminary studies using 50 mM taurine were aborted due to the lungs filling with fluid.

From these results, it appears that taurine was not able to protect the lung at the relatively low concentrations tested (5 and 10 mM). However, the data shown in Tables 1-3 is variable and should be interpreted with caution. If accurate, the inability of taurine to protect the lung from ischemia-reperfusion injury at low concentrations is consistent with results obtained in the in vitro experiments where concentrations of 50 to 300 mM taurine were required to achieve significant competition for free radicals. Taurine may not have been able to protect the lung because its free radical scavenging capacity was not significant enough at the concentrations tested to demonstrate an effect.
The nature of the ischemia-reperfusion injury process is very complex and involves many processes. The fact that taurine was ineffective at inhibiting free-radical induced lipid peroxidation may be an important part of the explanation for taurine's inability to protect against oxidative pulmonary injury because lipid peroxidation processes are believed to be involved in ischemia-reperfusion injury. Additionally, the fact that taurine was found to stimulate activated macrophages into producing increased amounts of H$_2$O$_2$ may also be significant in the lung injury model because the role of macrophages stimulated into respiratory burst, which may be present and producing reactive oxygen species during the injury process, has not yet been determined. Components other than those mediated by reactive oxygen species are believed to play significant roles also. These other processes, which are believed to involve cellular mediators of inflammation, may have masked the free radical scavenging effect of taurine. While it is interesting to speculate why taurine may not have had a protective effect in the ischemia-reperfusion injury model, it should be emphasized that the data collected were variable and further study is warranted.
Chapter 5

CONCLUSION

The data obtained in these studies provide important information about the mechanism by which taurine and hypotaurine exert their antioxidant effects, however, the data also leave many questions unanswered. The fact that both taurine and hypotaurine were able to demonstrate the ability to scavenge the potent hydroxyl radical indicates that each of the amino acids possesses antioxidant properties. However, the addition of either of the two amino acids to lungs exposed to oxidative injury offered insignificant protection and at some concentrations may have been toxic to the lungs.

In conclusion, the antioxidant properties of taurine and hypotaurine are due to their capability to scavenge some of the reactive species of oxygen. The inability of low concentrations of taurine to ameliorate post-ischemic reperfusion injury of lungs is consistent with the fact that relatively high concentrations of taurine were needed for the amino acid to demonstrate significant scavenging of $O_2^-$ and OH. These findings suggest that lower concentrations of taurine (5-10 mM) would not provide a therapeutic advantage in treating oxidative lung injury and that higher doses might be toxic.
REFERENCES


VITA

Carroll Moses Garland was born on June 16, 1966 in Pearisburg, Virginia and is the daughter of Buddy and Becky Moses of Pearisburg. She graduated from the College of William and Mary in May of 1988 with a Bachelor of Science degree in Biology. Carroll moved to Evanston, Illinois in June of 1988 and began work as a Research Technologist in the Department of Biochemistry, Molecular Biology and Cell Biology at Northwestern University. In 1989, she moved back to Virginia and accepted a position at the University of Virginia Medical Center as a Laboratory Specialist in the Department of Pathology. In order to pursue her interest in veterinary medicine, Carroll accepted a position as an Equine Veterinary Assistant at Virginia Equine Clinic in January of 1991 and she worked there until July of 1991 when she entered Virginia Tech as a candidate for a Master of Science degree in Biochemistry and Nutrition. Carroll transferred her candidacy to that of Master of Science in Veterinary Medical Sciences in August of 1992 when she began working under the supervision of Dr. Hara Misra at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM). In the fall of 1994, Carroll entered the Doctorate of Veterinary Medicine program at VMRCVM. On October 1, 1994 she was married to David S. Garland of Richmond, Virginia. Carroll is a candidate for graduation from the DVM program in May of 1998.

Carroll Moses Garland

HONORS AND AWARDS

Graduate Teaching Assistantship
Phi Sigma Biological Honor Society
Morris Animal Foundation

March 17, 1992, Selected for membership
Selected as Goodwill Ambassador for
VMRCVM April '95-April '97

ABSTRACT/PRESENTATION

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