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A Mechanistic Study of Myoglobin Nephrotoxicity

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A MECHANISTIC STUDY OF MYOGLOBIN NEPHROTOXICITY

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Myoglobin is an endogenous protein that can become nephrotoxic under certain conditions such as crush injuries, drug overdose, and seizures where prolonged contraction of muscle leads to cell death and leakage of myoglobin. The mechanism of myoglobin-induced nephrotoxicity is not fully understood. The purpose of this study was to characterize the sequence and mechanistic events associated with the *in vitro* toxicity of myoglobin in renal cortical slices. Renal tissue was isolated from Fischer 344 rats. Slices of renal cortex were prepared by freehand. These slices were then incubated for 60-180 minutes with myoglobin (0-12 mg/mL) pretreated with 4 mM ascorbic acid. Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release, pyruvate-stimulated gluconeogenesis, and lipid peroxidation. In addition, glutathione (total and oxidized) and ATP levels were measured. Toxicity was evident at one hour by changes in gluconeogenesis, lipid peroxidation, and glutathione levels. LDH release and a decline in ATP levels were not observed until two hours of incubation with myoglobin. In short, oxidative events (namely lipid peroxidation and changes in glutathione levels) and deficit of cell function (decreased gluconeogenesis) preceded loss of viability by one hour.

Pretreatment of the slices with deferoxamine (DFX) afforded protection against oxidative events and loss of membrane integrity, but not of the decrease in cell function. This finding suggested an early bifurcation in the toxicity pathway with loss of cell function residing in one path and iron-dependent oxidative events followed by loss of membrane integrity in the other. Pretreatment of the slices with exogenous reduced glutathione provided

protection of all toxic events, suggesting an underlying oxidative mechanism for the loss of gluconeogenesis that is iron-independent. Furthermore, the lack of protection against LDH release and loss of gluconeogenesis by pretreatment with dimethylthiourea indicated an absence of hydroxyl radicals in the mechanism of myoglobin toxicity in the slice model. Similar to DFX, pyruvate induced a general increase in the total glutathione levels and protection against lipid peroxidation. However, in contrast to DFX, pyruvate did not provide protection against the myoglobin-induced decline in total glutathione levels with respect to the control group. The protection provided by pyruvate appears to involve detoxification of radical pathways.

A comparison of the toxicity of myoglobin with its components suggested that the iron is mostly involved with the loss of membrane integrity and slightly involved with the loss of cell function. Because DFX can detoxify ferryl myoglobin as well as chelate free iron, the protection of lipid peroxidation, changes in glutathione levels, and LDH release suggest that ferryl myoglobin also might participate in the oxidative events leading to loss of membrane integrity. In contrast, the heme portion of myoglobin might target the mitochondria, initiate the production of radicals, and lead to loss of cell function indicated by loss of gluconeogenesis.

In contrast to mitochondrial-substrate stimulation of gluconeogenesis, cytosolic-substrate stimulation of gluconeogenesis was not affected by the presence of myoglobin. Taken together, the experiments presented in this study suggest that myoglobin targets mitochondria and produces toxicity

predominately through oxidative damage. Moreover, this study establishes three unique events concerning myoglobin toxicity. First, early and late effects of myoglobin toxicity have been delineated. Other studies have reported LDH release, lipid peroxidation, and alterations in glutathione and ATP levels, but none has ever evaluated these parameters as a function of time. Secondly, this study establishes iron-dependent and iron-independent components of myoglobin toxicity. Lastly, because myoglobin toxicity was demonstrated in a renal model that has collapsed lumens, this suggests toxicity can occur independently of luminal events.

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INTRODUCTION

Myoglobin is a 17kD protein consisting of eight α-helices conjugated to a heme group. The synthesis of myoglobin involves production of the protein portion and the heme portion separately. The construction of heme occurs in the cytosol and the mitochondria with the final steps encompassing the insertion of iron into protoporphyrin IX by ferrochelatase. In muscle cells, the iron in the heme group is maintained in the reduced ferrous (Fe^{2+}) state (Moore *et al.*, 1998). However, it is estimated that at any given time *in vivo*, 1-2% of human hemoglobin is in the ferric (Fe^{3+}) form (Antonini and Brunori, 1971). This ferric form is capable of redox cycling to a ferryl form ($[Fe=O]^{2+}$) which can initiate lipid peroxidation (Hogg *et al.*, 1994 and Patel *et al.*, 1996). Human myoglobin is similar in sequence to other mammalian myoglobins with the exception of a unique cysteine at position 110. No other species of known myoglobin has a cysteine residue (Hubbard *et al.*, 1990.)

Myoglobin is found in cytosol of muscle cells in varying concentrations with smooth muscle having virtually none (except the uterus) and skeletal muscle averaging 0.5mmole per kg tissue weight (Wittenberg, 1970). Diving animals have up to 10-fold more myoglobin which is thought to provide an extension of diving time when pulmonary ventilation ceases (Guyton *et al.*, 1995). In cardiac muscle, myoglobin serves as a short-term oxygen reservoir by supplying the muscle with oxygen from one contraction to the next (Millikan, 1937). Myoglobin and hemoglobin play a vital role in the acquisition and utilization of oxygen. Specifically, myoglobin is proposed to deliver oxygen to the mitochondria (Wittenberg, 1970). Studies with transgenic mice lacking myoglobin surprisingly resulted in a benign phenotype with exercise, reproductive, cardiac, and

skeletal capabilities largely unaltered (Garry *et al.*, 1998). This was accomplished by maintenance of function by various compensating mechanisms such as an increase in vascularization of the muscle, coronary blood flow, and hematocrit (Gödecke *et al.*, 1999).

Rhabdomyolysis by definition is the injury of muscle with release of myoglobin and other cellular contents. Rhabdomyolysis may occur in various circumstances including: crush injuries; fallen elderly who have lain for several hours; drug abuse; physical abuse such as spouse or child abuse; and strenuous exercise such as marathon running and football. In fact, 39% of the Marine Corps recruit population in the 70's exhibited symptoms of rhabdomyolysis (Olerud *et al.*, 1975). This is of no surprise since skeletal muscle is the largest organ in the body, comprising 40-50% of total body weight (Abassi *et al.*, 1998).

The sarcolemma (the membrane of the muscle cells) is normally impermeable to the extracellular fluid (ECF), protecting the cytosol by maintaining cellular contents and ion balance (Better *et al.*, 1992). Generally, minor sarcolemma disruptions are compensated for by Na^+-K^+-ATPase pumps that maintain the electronegativity of the cell by sequestering potassium ions and extruding sodium ions. However, excessive sarcolemma damage causes an overwhelming two-way leak. Calcium ions, sodium ions, and water leak into the cell causing depletion of ECF while potassium ions, phosphates, purines, and myoglobin leak out of the cell (Better *et al.*, 1997). The myoglobin that spills into the ECF enters the blood and is readily filtered by the kidney (Ratcliffe *et al.*, 1986). When the filtered load exceeds the absorptive capacity, then myoglobin can precipitate in the tubular lumen and subsequently release iron into the

lumen (Bunn and Jandl, 1969 and Zager, 1992). Both the iron and the myoglobin are nephrotoxic. In fact, it is estimated that a third of all rhabdomyolysis patients will develop acute renal failure (ARF) (Gabow *et al.*, 1982). In addition, rhabdomyolysis accounts for 5-7% of all ARF cases- certainly no trivial matter (Grossman *et al.*, 1974).

Overall, the total manifestation of myoglobin toxicity can be described as three basic events: vasoconstriction, tubular obstruction, and pigment nephropathy. A decrease in renal perfusion is consistently reported in experimental models of myoglobin-ARF (Zager, 1996b; Vetterlein *et al.*, 1995). The vasoconstriction component is thought to be due to several factors including release of endothelin. Endothelin is a potent renal vasoconstrictor and serum levels were found to be elevated in rhabdomyolysis (Kohan, 1997; Brooks, 1997). Also, isoprostanes, other potent renal vasoconstrictors, were found to be elevated in the urine of patients with rhabdomyolysis (Holt *et al.*, 1999). Besides the direct effect of vasoconstrictors on the kidney, myoglobin has the ability to chelate nitric oxide (NO) and to induce nitric oxide synthase (iNOS) (Lieberthal, 1997; Rubinstein *et al.*, 1998). This chelation of a vasodilatory factor would potentiate the induced vasoconstriction resulting in decreased glomerular filtration rate (GFR). The reduction in GFR can hasten the tubular obstruction component of myoglobin toxicity. The tubular obstruction occurs in the distal tubules and arises from the deposition of the ferric (Fe^{3+}) redox form of myoglobin (Moore *et al.*, 1998) in addition to the debris from necrotic proximal tubule cells. Together, these precipitates give rise to a heme cast in the distal nephron with the blockage further hindering urine flow through the nephron. Aside from the contributing hemodynamic events such as vasoconstriction and tubular obstruction, myoglobin itself is toxic to

proximal tubular cells. This component of the toxicity is often termed pigment nephropathy. Upon microscopic examination of renal tissue from subjects with rhabdomyolysis, necrosis occurred specifically in the S3 segment (Heyman *et al.*, 1997; Zager, 1989) in addition to prominent brush border injury (Zager, 1989) and detachment of epithelial cells from the basement membrane (Zager, 1996b).

Treatment of rhabdomyolysis begins with massive replacement of fluids, 12 L per day. Sodium bicarbonate is given to reverse the metabolic acidosis and to reduce the toxic effect of the myoglobin. Reportedly, the entire 14 L of ECF can be sequestered into crushed muscles within hours of injury (Schaller *et al.*, 1990). Mannitol is administered to osmotically pull the excess water from the injured muscle. This reduces the intracellular volume as well as replenishes the ECF. Mannitol has an additional role of increasing the GFR, thus increasing urine flow and decreasing the precipitation of proteins. Generally, with treatment, urinary myoglobin disappears after three days. In the case of crush injuries, if treatment is started within six hours of injury, myoglobin-induced ARF can be completely prevented even in those buried for up to 32 hours. This drastically contrasts with the 100% mortality associated myoglobin-ARF in those extricated after 3-4 hours in London during World War II (Abassi *et al.*, 1998).

Few experimental models have been used to investigate myoglobin toxicity. The most widely applied model of myoglobin-ARF is the glycerol *in vivo* model. Following a 24-hour water deprivation, animals are injected into the hind limb with a hypertonic solution of glycerol (generally 10 mL per kg body weight). After injection into muscle, virtually all rats develop ARF and a urine concentration of 35 mg/mL of myoglobin is typically observed (Zager, 1989; Zager *et al.*, 1991). At various times post-injection (4-

24 hours), the toxicity was assessed. Many of the experiments use the 4-hour post-glycerol injection time. At this time point, cell injury is noted by brush border blebbing. However, necrosis remains to be seen until a later time point (Zager *et al.*, 1995). Zager and associates often use a hybrid model referred to as an *in vivo-in vitro* model. In this model, the animals are injected with glycerol followed by an isolation of the proximal tubules for further incubations. Aside from the glycerol model, another *in vivo* model is the perfusion model where myoglobin is directly perfused into the animal kidney. Heyman *et al.* (1997) reported a marked decrease in GRF and a three-fold increase in plasma creatinine accompanied by a 30% drop in creatinine clearance following perfusion. Previous *in vitro* studies have used both freshly isolated proximal tubules and cultured HK-2 cells. HK-2 cells are immortalized proximal tubule cells derived from normal human kidney. Incubations are typically performed over 24 hours. Because of different experimental conditions with the different models, there is much variability among the findings. Despite this, a few themes have prevailed throughout the years.

One hypothesis concerning the mechanism of myoglobin toxicity is the involvement of free radicals. One of the classic findings concerning myoglobin toxicity is the elevated level of H_2O_2. In fact, the glycerol model has been used to study various aspects of H_2O_2 toxicity (Salahudeen *et al.*, 1991). H_2O_2 can liberate iron from the heme proteins (Gutteridge, 1986). This iron could have a critical role in the formation of free radicals. Other findings suggest that indeed, a radical mechanism is involved. Shah and Walker (1988) reported that rats treated with glycerol produced increased malondialdehyde levels, indicative of lipid peroxidation. In addition, Moore *et al.* (1998)

reported an increase in urinary excretion of F_2-isoprostanes in rats with rhabdomyolysis. More specifically, the levels of F_2-isoprostanes esterfied in renal lipids were markedly increased which localized oxidant injury to the kidney. This provided more evidence that lipid peroxidation in the kidney is a feature of myoglobin toxicity.

The protection afforded by deferoxamine, a free Fe^{2+} chelator, lends more support to the free radical hypothesis. Deferoxamine (DFX) has been shown to decrease renal injury in the glycerol model and to prevent cytotoxicity of myoglobin on cultured proximal tubule cells *in vitro* (Paller, 1988; Zager and Burkhart, 1997). The protective effect of iron chelators, such as DFX, has generally been taken as evidence for participation of hydroxyl radicals. However, none of the studies agreed on the involvement of hydroxyl radicals. Some studies suggested that the ferryl redox form of the myoglobin was the main culprit (Holt *et al.*, 1999; Hogg *et al.*, 1994; Patel *et al.*, 1996).

The mitochondria also have been implicated in the formation of free radicals. In rats three hours post-glycerol injection, impaired respiration was detected in mitochondria; whereas, at 24 hours post-glycerol injection, respiratory failure was observed (Nath *et al.*, 1998). Zager (1996a) implicated the terminal mitochondrial transport chain as the origin of free radicals that initiated injury; in effect, stating that the mitochondria was the site of initial insult. He further concluded that the mitochondrial transport chain was a critical mediator in myoglobin induced cell death in addition to being a determinant of lipid peroxidation (Zager and Burkhart, 1997). If the mitochondria were indeed targets for myoglobin toxicity, then it would be logical to detect drops in ATP levels prior to loss of viability. Trifillis *et al.* (1981) showed a

decrease in ATP content in renal cortex of rats with glycerol-induced rhabdomyolysis. Zager (1996a) also reported a decline in ATP levels, albeit accounted for by increased cell membrane permeability as measured by LDH release. Thus, the mitochondria may be targeted in a way that does not rapidly deplete ATP levels.

Several studies have investigated the glutathione system in response to myoglobin challenge. Abul-Ezz *et al.* (1991) reported that injection of glycerol in rats produced a significant and early depletion of renal glutathione. Total renal glutathione was diminished below control values within one hour post-injection. Unfortunately, no rise in glutathione disulfide levels was noted as these levels decreased in parallel to total glutathione levels. In addition, administration (i.v.) of reduced glutathione led to a significant overall improvement of renal function as measured by blood urea nitrogen (BUN) concentration, creatinine levels, and histological examination by light microscopy. Likewise, depletion of glutathione with L-buthionine-(S,R)-sulfoximine (BSO) significantly exacerbated toxicity. Zager *et al.* (1995) reported similar findings in the glycerol-injection, *in vivo-in vitro* model. Reduced glutathione was directly incubated with post-glycerol, isolated proximal tubule segments. A weak cytoprotection as measured by LDH release was noted. However, the incubation with glutathione markedly increased lipid peroxidation as indicated by a dramatic rise in malondialdehyde levels. Zager and Burkhart (1998) further investigated the role of exogenous glutathione administration using the HK-2 cell culture model. Incubation of the HK-2 cells with 4 mM glutathione before or during the 24-hour myoglobin (10 mg/mL) challenge resulted in increased LDH release. In addition, glutathione depletion

via BSO conferred cytoprotection. Overall, the role of glutathione in the mechanism of myoglobin toxicity is incompletely understood.

It cannot be overstated that many discrepancies (such as radical identity and glutathione involvement) in the data exist in previous studies depending upon the models used. These discrepancies could be explained by the complexities of the models. For example, the glycerol *in vivo* model has the unfortunate problem of hemodynamic events affecting the results. Isolated and cultured cell models pose several potential problems. Both in *vitro* models involve a non-intact environment due to the anatomical destruction of the nephron. The freshly isolated cells give rise to the question of true viability. It could be possible that cell selection inadvertently occurs when the most injured cells are not 'selected' during the isolation protocol. The immortalized, cultured cells are altered in a way that can affect metabolism, transport, and mechanisms of toxicity in those cells.

The *in vitro* model used in this study is a renal cortical slice model. This model was chosen for its multiple advantages over the previously used models. Unlike the *in vivo* models, the slice model would eliminate any confounding factors owing to hemodynamic events, such as the release of endothelin and/or elevation of isoprostanes. In contrast to cell cultures, the slice model provides an intact cortical nephron to study the toxicity. Although the lumen in the nephron is collapsed, this is advantageous as it allows one to assess whether myoglobin must enter through the brush border, which has been suggested as a route of entry (Clyne et al., 1979). Because in this model the lumen is inaccessible, this question would be resolved. Male Fischer 344 rats were used in this study because they are more sensitive to renal

toxicants (Kacew *et al*., 1995) and often react comparably to renal toxicants, as humans do. Male rats were used to eliminate any confounding results due to cycling of hormone levels.

Several measures of toxicity were employed in this study including: lactate dehydrogenase (LDH) release, gluconeogenesis, lipid peroxidation, glutathione levels (total and glutathione disulfide), and ATP levels. LDH release is indicative of irreversible cell damage (Lash and Tokarz, 1989) and was used to assess viability of the tissues when exposed to myoglobin for various times. Gluconeogenesis is an informative measure of toxicity for two fundamental reasons. In the kidney, the proximal tubules contain high levels of enzymes for gluconeogenesis with low levels of enzymes for glycolysis (Guder and Ross, 1984; Gesek *et al*., 1987; Schoolwerth *et al*., 1988; Lash and Tokarz, 1989). Thus, within the kidney, gluconeogenesis occurs exclusively in the proximal tubule cells and any decreases in gluconeogenesis could be extrapolated to injury specifically to the proximal tubules. Furthermore, gluconeogenesis is a very sensitive marker for toxicity, as changes in gluconeogenesis can be detected early and can indicate changes in cell function. In addition, the stimulation with cytosolic or mitochondrial substrates can help to elucidate the site of toxicity by allowing a partial determination of the cellular location of dysfunction. Further studies examined lipid peroxidation and changes in glutathione levels to assess the involvement of a radical mechanism. Lastly, ATP levels were measured to characterize alterations in adenine nucleotides as a function of myoglobin concentration and duration of exposure. The five parameters of toxicity aforementioned were used to characterize

myoglobin toxicity in the *in vitro* slice model as well as to set up a time frame for mechanistic events.

In the early 1940's, Bywaters and Bealle (1941) gave the classic definitive description of crush syndrome. They described the precipitation of myoglobin in the tubules and its presence in the urine. However, it was not until 1944 that myoglobin was first incriminated, albeit not exclusively, as the causative agent in the development of acute renal failure following crush injuries (Bywaters and Stead, 1944). Myoglobin toxicity is not a trivial affair. As stated before, rhabdomyolysis accounts for 5-7% of all ARF cases (Grossman *et al.*, 1974). In addition, globin nephrotoxicity is the main obstacle in the development of therapeutic blood substitutes (Pool, 1990). Since the 1940's, scientists have sought to uncover the underlying mechanism of myoglobin (and other globins) toxicity. Nearly sixty years later, the definitive mechanism is still unclear. This study seeks to characterize myoglobin toxicity in the rat renal cortical slice model and use the information to develop the time frame for mechanistic events.

CHAPTER 1. CHARACTERIZATION AND BASIC MECHANISM OF MYOGLOBIN TOXICITY IN RENAL CORTICAL SLICES

1.1. INTRODUCTION

Myoglobin toxicity has been studied for almost 60 years. Despite this, many unanswered questions remain concerning the mechanism of toxicity. Most studies reported that both reactive and non-oxygen based radicals are critical in the pathogenesis of various renal diseases including Myoglobin-ARF (Zager, 1996b) as evidenced by increased lipid peroxidation and depletion of glutathione. Elevated levels of H_2O_2 can liberate iron from the heme proteins (Gutteridge, 1986) and this free iron, in addition to being toxic, may have a critical role in the formation of radicals. The protection afforded by deferoxamine (DFX), a free Fe^{2+} chelator, lends more support to the free radical hypothesis as it is often associated with iron-dependent radicals such as hydroxyl radicals. In addition to chelating free iron, DFX can detoxify the ferryl form of myoglobin (Turner *et al*., 1991) and some studies suggested the ferryl redox form of the myoglobin is a major radical in myoglobin toxicity (Holt *et al.*, 1999; Hogg *et al.*, 1994; Patel *et al.*, 1996). Taken together, clearly there is an underlying radical mechanism in myoglobin toxicity.

The mitochondria have been implicated in the formation of oxygen-based free radicals and as a site of myoglobin toxicity. Using mouse proximal tubular segments (PTS) and the HK-2 cell model, Zager (1996a; *et al.,* 1997) implicated the terminal mitochondrial transport chain (notably site 3) as the origin of free radicals that initiate injury and later concluded that the mitochondrial transport chain was a critical mediator in myoglobin induced cell death in addition to being a determinant of lipid peroxidation.

Later in 1998, Nath *et al.* measured a ten-fold increase in heme content in the mitochondria three hours post-glycerol injection into rats, at a time when impaired respiration was detected in mitochondria.

Several studies have investigated the glutathione system in response to myoglobin challenge. Glutathione is a tri-peptide occurring in high concentrations in virtually all mammalian cells and is the most prevalent intracellular thiol (Meister, 1983, 1984a, 1984b, 1986). Glutathione exists in both the cytosolic and mitochondrial compartments with mitochondrial glutathione accounting for approximately 10% of total glutathione. Glutathione metabolism in the kidney is distinguished from metabolism in other organs by rapid turnover (Sekura and Meister, 1974; Rankin *et al.*, 1985). The protective effect of glutathione in oxidant injury to freshly isolated proximal tubule cells has been shown in several *in vitro* studies with other toxicants (Lash and Tokarz, 1990; Messana *et al.*, 1988; Hagen *et al.*, 1998).

As outlined before, many discrepancies in previous findings exist which may be explained by the underlying complexities of the different models used to study myoglobin toxicity. In this study, the model used is the renal cortical slice model with its many advantages over the other models as previously detailed. This chapter will characterize the *in vitro* toxicity of myoglobin to renal cortical slices. Following myoglobin incubation with the slices, several assays were performed to assess the toxicity. LDH release was used to determine membrane integrity and gluconeogenesis was employed to investigate early cell function. Lipid peroxidation and glutathione levels were measured to address oxidative events. The amount of ATP was used to evaluate energetics. The incubations were performed with various incubation times in

order to place each measure of cytotoxicity within a time frame. Tangent experiments investigated possible targets for toxicity and initial radical formation.

Protection assays were used to further probe the basic time frame of toxicity for mechanistic events. A separate series of experiments examined the effect of an iron chelator (DFX), α-ketoacid (pyruvate), and a thiol-containing agent (reduced glutathione). In previously published studies by other investigators, iron chelators, such as DFX, have been consistently found to protect against myoglobin toxicity. Reproducing this protection in our model would validate our model. Furthermore, investigation of the DFX protection at various time points would give deeper insight to the mechanism of myoglobin toxicity. Pyruvate was chosen for the protection assays for two basic reasons. Because it was used to stimulate gluconeogenesis, pyruvate could potentially affect interpretation of the data if it provided any protection or detriment. In addition, pyruvate has the capacity to detoxify radical pathways. Therefore, the use of pyruvate would suggest which time points could involve radical mechanisms. Exogenous reduced glutathione (GSH) application was used to further investigate the mechanism of toxicity and to complement examination of glutathione levels following myoglobin exposure.

1.2. MATERIALS AND METHODS

1.2.1 Chemicals / equipment and animals

Chemicals / equipment and sources
(Company and catalog number)

Horse skeletal muscle myoglobin (Sigma, M-0630)

Ascorbic acid (Fisher Scientific, A-61)

Deferoxamine mesylate (Sigma, D-9533)

Reduced glutathione (Sigma, G-6529)

Glutathione disulfide (Sigma, G-6654)

2-vinylpyridine (Aldrich, 13229-2)

Lactate dehydrogenase kit (Sigma, 228)

Infinity glucose reagent (Sigma, 18)

Pyruvic acid (Sigma, P-2266)

d-Glucose-6-phosphate (Sigma, G-7879)

d-Fructose-1,6-biphosphate (Sigma, 752-1)

Malondialdehyde (Aldrich, 12960-7)

2-Thiobarbituric acid (Sigma, T-5500)

Adenosine 5' triphosphate (Sigma, A2383)

Glutathione reductase (Sigma, G-3664)

0.45 μm syringe filter (Durapore; Millex-HV)

Radial-Pak C18 cartridge (8mmx10 mM) (Waters Inc.; Milford, Massachusetts).

5,5'-dithiobis(2-nitrobenzoic acid) (Aldrich, D21820-0)

Cytochrome C

Dubnoff metabolic shaker (Precision Scientific; Chicago, IL)

Sorval MC12V centrifuge

Animals

Male Fischer 344 rats (200-250 g) were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA, USA). Animals were maintained under a controlled ambient temperature (21-23^{o}C), humidity (40-55%) and light cycle (lights on 0600-1800 h). Animals were provided free access to tap water and Purina Rat Chow (chunks). All rats were given a minimum 5-day acclimation period prior to initiation of any experiments. This was the only animal model used throughout this study.

1.2.2 Incubation of renal slices.

Animals were anesthetized with diethyl ether. The abdominal aortae were cut to exsanguinate the animals. The kidneys were decapsulated, excised, quartered and immediately placed in 5 mL ice-cold Krebs Ringer buffer kept on ice. Renal cortical slices were prepared freehand as described previously (Valentovic *et al*., 1992) and placed in 10 mL ice-cold Krebs-Ringer buffer. The slices from one animal were transferred to 5 mL oxygenated Krebs-Ringer buffer in a 30-mL beaker. The slices were rinsed two times in 5 mL oxygenated Krebs-Ringer buffer each for three minutes at 25^{o}C in an oxygen environment with constant shaking (100 cycles/minute) in a gabled Dubnoff metabolic shaker. The tissue (50-100 mg) was evenly distributed in 2 mL of oxygenated Krebs in designated Erlenmeyer flasks and equilibrated for 10 minutes at 37^{o}C under 100% oxygen and constant shaking (100 cycles/minute). Renal tissue was incubated for 60-120 minutes with a final concentration of 0, 4, 10 or 12 mg/mL myoglobin added as a 1 mL aliquot with a total incubation volume of 3 mL (see Appendix I for preparation of myoglobin solution). Gluconeogenesis was stimulated by

the addition of pyruvate (100 μL, final bath concentration 10 mM) and tissues were incubated an additional 30 minutes. Media and tissue were collected to measure LDH leakage and glucose generation.

Other experiments required a pretreatment of renal slices at 37°C with 30 μL distilled water, 1mM reduced glutathione (GSH), or 0.1mM deferoxamine. The durations of pretreatments were 30 minutes for glutathione and 15 minutes for deferoxamine. Upon completion of the pretreatment period, myoglobin was added at a final bath concentration of 0, 4, 10 or 12 mg/mL. Tissues were incubated for 90 minutes at 37°C under 100% oxygen and constant shaking (100 cycles/minute). At the end of the 90-minute incubation, gluconeogenesis was stimulated by addition of 100 μL of pyruvate (10 mM final concentration, in Krebs) and the tissue was incubated an additional 30 minutes.

Additional studies were conducted with glutathione to ensure that no physical interaction was occurring extracellularly between myoglobin and glutathione. Renal slices were pretreated for 30 minutes with 1mM glutathione at 37°C under a flow of 100% oxygen and constant shaking. The tissues were then removed from the media and placed in fresh buffer without glutathione. Myoglobin (final concentration 0, 4, 10 or 12 mg/mL) was added and tissues were incubated for 90 minutes, followed by addition of pyruvate (10 mM final concentration) and incubation of the tissues for an additional 30 minutes.

Because pyruvate was used to stimulate gluconeogenesis, additional studies addressed the possibility of any influence on the resulting toxicity. Renal slices (50-100 mg) were incubated with pyruvate (10 mM final concentration) and myoglobin (final

concentration 0, 4, 10 or 12 mg/mL) for two hours at 37^{o}C under 100% oxygen and constant shaking (100 cycles/minute).

1.2.3 LDH and glucose assays.

Upon completion of incubation, tissues were blotted, weighed and added to 10% Triton X-100 in buffer to release tissue LDH. The amount of LDH in the media and tissue was determined using a spectrophotometric kinetic assay (Sigma, Kit #228). LDH release into the media was expressed as percent of total LDH. Experiments in which control tissue LDH leakage exceeded 15% were discarded and not used in the final data analysis.

Glucose was measured in the media using a hexokinase enzymatic assay (Sigma, Kit #18). Myoglobin standards were done in order to calculate the background absorbance due to myoglobin from the samples. For all gluconeogenesis data, the background was subtracted. Pyruvate-stimulated gluconeogenesis was expressed as mg glucose/g tissue.

1.2.4 Adenine nucleotides.

Upon completion of incubation, renal tissue was blotted, weighed and homogenized in 1mL of Krebs-Ringer buffer. The homogenate was vortexed and 250 μL was combined with 125 μL of 3 N perchloric acid. The sample was vortexed and allowed to equilibrate for five minutes. The samples were centrifuged for 10 minutes at 2000 g in a Sorval MC12V centrifuge. A 300 μL aliquot of supernatant was removed and adjusted to pH 7 with KOH. The samples were vortexed, centrifuged for 10 minutes

at 2000 g. The supernatant was filtered through a 0.45 μm syringe filter. The levels of ATP were determined using an HPLC method adapted from Lash and Jones (1996). A 100 μL aliquot of sample was injected into a Beckman 126 model HPLC with a 100 μL injector loop equipped with a Beckman 166 variable wavelength detector. The mobile phase was a gradient of two solutions: Solvent A: 100 mM potassium phosphate, pH6.0 and Solvent B: methanol. The gradient was 7.5 minutes at 100%A / 0%B; 7.5-minute linear gradient to 90%A / 10%B; 15-minute linear gradient to 75%A / 25%B; 0.5-minute linear gradient to 100%A / 0%B; 10-minute re-equilibration at initial conditions. The flow rate of the mobile phase was 1.3mL/minute. The column was a Radial-Pak C18 cartridge (8 mm x 10 mM). The wavelength for detection was 254 nm. ATP values were calculated from a standard curve of ATP (0.94 to 9.4 nmol). ATP levels as low as 1nmole could be detected and were linear with respect to the standard curve. In addition, a set of standards was treated as samples to ascertain the extraction efficiency. The efficiency was determined to be approximately 80%.

1.2.5 Glutathione determination.

Renal cortical slices (50-100 mg) were incubated with 0, 4, 10 or 12 mg/mL myoglobin for 60, 90 or 120 minutes, as described above. Tissues were weighed and then homogenized in 0.5% sulphosalicylic acid in a 1mL total volume. Total glutathione was determined by an enzymatic reaction with glutathione reductase using 5,5'-dithiobis(2-nitrobenzoic acid) and NADPH (Tietze, 1969; Andersen, 1985). Glutathione disulfides (GSSG) were measured on samples derivatized with 2-vinylpyridine (Griffith,

1980) prior to enzymatic measurement of glutathione. Values were expressed as nmol/g tissue. See Appendix I for detailed methods.

1.2.6 Lipid peroxidation.

Renal cortical slices were prepared and equilibrated as described above. Renal slices were incubated in 3 mL oxygenated Krebs-Ringer buffer in an oxygen atmosphere and constant shaking (100 cycles/minute) at 37^{o}C. Lipid peroxide generation was measured in renal slices exposed to 0, 4, 10 or 12 mg/mL myoglobin for 60, 90 or 120 minutes. The slices were blotted, weighed and homogenized in 1mL Krebs-Ringer buffer. The homogenizer probe was rinsed with 1mL Krebs-Ringer buffer and adjusted to 2 mL. A 1.5 mL aliquot of the homogenate was added to an equivalent volume of 15% trichloroacetic acid dissolved in 0.25 N HCl. Protein was precipitated for 15 minutes on ice, and the supernatant was collected following centrifugation (2000 g at 4^{o}C, 10 minutes). The supernatant was heated for 15 minutes at 90^{o}C with an equal volume of 0.67% thiobarbituric acid (Ueda and Shah, 1996). The absorbance was measured at 535 nm, and the amount of malondialdehyde (MDA) was calculated based on a standard curve using MDA (0-40 nmoles) and expressed as nmol MDA/g tissue.

1.2.7 Microsome incubation with myoglobin

Liver microsomes were isolated from male Fischer 344 rats (see Appendix 1 for isolation protocol). To each 30 mL beaker, the following was added: 250 μL phosphate buffer or microsomes; 10 μL glucose-6-phosphate dehydrogenase; 750 μL myoglobin; 2 mL NADPH generating system (2 mM glucose-6-phosphate, 13 mM NADP). Following

incubation for 15 minutes at 37°C, the samples were combined with 250 μL of cytochrome C. The absorbance at 549 nm was recorded at various time intervals (0-90 sec). A change in absorbance would indicate a conversion of NADPH to NADP and thus detoxification of a radical.

1.2.8 Statistical analyses

(Sokal and Rohlf, 1969)

All statistical calculations were performed using SigmaStat software package (version 2.03). Values were reported as mean ± SEM. Paired t-tests were used to analyze the microsomal data. The remaining experiments were analyzed using a one-way analysis of variance (ANOVA) or a one-way repeated-measures analysis of variance (RM-ANOVA). Differences between groups within a treatment were analyzed using a repeated-measures analysis of variance (RM-ANOVA) followed by a Newman-Keuls test at a 95% confidence interval. To compare differences between treatments, an analysis of variance (ANOVA) followed by a Newman-Keuls test at a 95% confidence interval was used. All groups consisted of 4-7 animals.

1.3. RESULTS

1.3.1 Concentration-response study

Myoglobin was toxic when added to renal cortical slices. The concentrations of myoglobin selected initially were 1-12 mg/mL. These concentrations were relevant to levels reported in the literature for plasma levels measured in individuals with rhabdomyolysis (Hamilton *et al*., 1989; Shigemoto *et al*., 1997). Myoglobin induced concentration and time-dependent changes in renal cortical slices. Myoglobin produced a concentration-dependent loss of membrane integrity as indicated by an increase in the percentage of LDH release (Figure 1), which was seen with concentrations as low as 2 mg/mL myoglobin. Because concentrations of 0, 4, 10, and 12 mg/mL myoglobin produced the best concentration-dependent effect, these concentrations were chosen as the test concentrations for the remainder of the study.

As in other labs, myoglobin was pretreated with ascorbic acid to achieve the ferrous (Fe^{2+}) form, as this valence is reportedly the toxic form (Zager and Foerder, 1992; Zager and Burkhart, 1997). In this study, omission of pretreatment of the myoglobin with ascorbic acid greatly affected the toxicity. LDH release failed to rise above baseline if the myoglobin was not pretreated with ascorbic acid (Figure 2). However, pretreatment of the myoglobin with the ascorbic acid once again produced a concentration-dependent increase in the LDH release. For the remainder of the study, myoglobin was pretreated with 4 mM ascorbic acid and all studies denoted as myoglobin-treated were performed in this manner.

1.3.2 Time-course study

In order to evaluate a time course for myoglobin toxicity, incubations were conducted for 60-120 minutes. Myoglobin required a two-hour incubation to increase LDH release ($p<0.05$) in a concentration-dependent manner (Figure 3). In addition to LDH release, gluconeogenesis was chosen as a second measure for evaluating the basic time frame for the development of toxicity. A significant, concentration-dependent loss of gluconeogenesis was observed within a one-hour myoglobin incubation (Figure 4). Notably, this was one hour prior to significant LDH leakage. As incubation times increased, gluconeogenesis was nearly abolished. In general, all tissue samples including controls, displayed a gradual decrease in gluconeogenesis. To eliminate the possibility that the decline was not simply myoglobin interference in the glucose assay, standard curves of glucose in the presence of varying concentrations of myoglobin were compiled. Myoglobin shifted the glucose curves upwards with respect to increasing concentrations. To accommodate this, the myoglobin background at each concentration was subtracted from the results. Moreover, as further evidence, the decline of levels of gluconeogenesis was more pronounced in the presence of myoglobin (Figure 4). Taken as a whole, the time study of myoglobin exposure to renal slices revealed loss of gluconeogenesis occurring as early as one hour, while significant LDH leakage did not occur until two hours.

The remaining three measures of toxicity, namely ATP levels, lipid peroxidation, and, glutathione levels, were employed to investigate further the time course and possible events of myoglobin toxicity. ATP levels remained unchanged from control at 60 and 90 minutes of incubation (Figure 5). Conversely, at two hours, the ATP levels

significantly ($p<0.05$) declined with a concentration-dependent trend in the myoglobin treated as compared to the respective control. This time frame coincides with significant LDH release by myoglobin.

In contrast to ATP levels, lipid peroxidation and changes in glutathione levels occurred at one hour. Lipid peroxidation, as measured by the amount of malondialdehyde per gram tissue, was elevated by myoglobin exposure of renal slices relative to control (Table 1). At each additional incubation time, lipid peroxidation levels continued to be significantly different in the treated group compared to control. However, no significant concentration dependence of this response was detected at any of the incubation times. At two hours, the highest myoglobin concentration yielded lipid peroxidation levels of ~140nmol/g tissue in the myoglobin treated tissue and ~114nmol/g tissue in the control group.

Similar to lipid peroxidation, myoglobin-induced changes in glutathione levels occurred prior to LDH release. At one hour, total glutathione levels, expressed as nmol per gram tissues, were significantly decreased in the myoglobin groups as compared to control (Table 2). No difference in total glutathione was detectable between the various myoglobin concentrations. Myoglobin treatment was associated with an overall decrease in total glutathione levels over time. The amount of glutathione disulfide (GSSG), as measured by %GSSG of total glutathione, did not significantly increase until 90 minutes (Table 2). At two hours, the high concentration of myoglobin (12 mg/mL) produced a two-fold increase in GSSG compared to control. Taken together, the measures of toxicity established a basic time course for myoglobin toxicity. Gluconeogenesis, lipid peroxidation, and changes in glutathione levels were detected

as early as one hour whereas LDH release and changes in ATP levels were detected at two hours.

1.3.3 Protection assays

Protection assays were used to probe the basic time frame, providing possible mechanistic events. Deferoxamine (DFX) pretreatment partially protected renal cortical slices from myoglobin toxicity. Pretreatment of renal cortical slices for 15 minutes with DFX, followed by a two-hour incubation with myoglobin resulted in a significant attenuation of LDH release (Figure 6). The levels of LDH release in the presence of myoglobin were not significantly different from control within the DFX treatment. Moreover, at 12 mg/mL myoglobin, a significant reduction in LDH release was evident as compared to myoglobin and vehicle-treated tissue. The gluconeogenesis data revealed an opposite finding. Pretreatment of the slices with DFX failed to protect against the loss of gluconeogenesis induced by myoglobin (Figure 7). The decrease in pyruvate-stimulated gluconeogenesis was indistinguishable between DFX and DFX-vehicle-treated groups.

Pretreatment of the slices for 30 minutes with exogenous reduced-glutathione (GSH), followed by a two-hour incubation, resulted in protection from myoglobin-induced LDH release and loss of gluconeogenesis. Myoglobin produced no significant increase in LDH release within the GSH pretreated group (Figure 8). At all levels except control, the amount of LDH release was significantly lower in the GSH pretreated as compared to tissue pretreated with vehicle. Furthermore, the pretreatment with GSH prevented the loss of gluconeogenesis (Figure 9). The glucose levels in the presence

of myoglobin were not significantly different within the GSH pretreated group. At the higher concentrations of myoglobin, there was a significant reduction in toxicity in the GSH-pretreated group as compared to the GSH-vehicle-pretreated group.

Because of the potential for GSH to detoxify myoglobin before it enters the cell and thus result in protection, a separate study with exogenously applied GSH was undertaken. Once pretreated for 30 minutes with GSH, the slices were removed and placed in fresh pre-warmed buffer lacking GSH. The renal slices were then incubated with myoglobin. The GSH and myoglobin were not permitted to incubate together in the extracellular media. Under these limiting conditions, GSH still provided remarkable protection. LDH release was not altered by myoglobin in the GSH pretreated tissue (Figure 10). Also, there was a significant difference in the highest concentration of myoglobin between the GSH + rinse treated versus the vehicle. Analogous results were obtained for gluconeogenesis (Figure 11).

Pyruvate treatment was chosen as another means to probe the mechanism of toxicity. With the concurrent treatment of pyruvate, LDH release in response to myoglobin was slightly increased above controls (Figure 12) in the pretreated group. In addition, in tissues exposed to 10 and 12 mg/mL myoglobin, LDH release was significantly lower in the pyruvate-treated group relative to the pyruvate-vehicle group.

1.3.4 Intracellular targets

Pyruvate was the selected substrate for mitochondrial stimulation of gluconeogenesis throughout this study. A separate set of experiments evaluated the effect of stimulating gluconeogenesis with cytosolic substrates, namely fructose-1,6-

diphosphate and glucose-6-phosphate. Evaluation of gluconeogenesis in the presence of 4 mg/mL myoglobin or control revealed different results between the cytosolic and mitochondrial substrates. Myoglobin had no detrimental effect on gluconeogenesis when it was stimulated with fructose-1,6-diphosphate or glucose-6-phosphate (Figure 13). This was in contrast to the stimulation with pyruvate that resulted in significant loss of gluconeogenesis.

1.3.5 Spontaneous radical formation

As delineated in the introduction, a free radical mechanism is thought to be responsible for myoglobin toxicity. A microsomal assay was conducted in order to ascertain if a free radical is spontaneously produced or if it requires activation by microsomal enzymes. The change in absorbance at 549 nm would indicate an oxidation of the NADPH to NADP and thus detoxification of a radical. In the absence of microsomes, 4 mg/mL myoglobin, as compared to control, failed to produce any change in the absorbance per minute (Table 3). The addition of microsomes had no effect. In fact, all levels (with and without microsomes) were not significantly different.

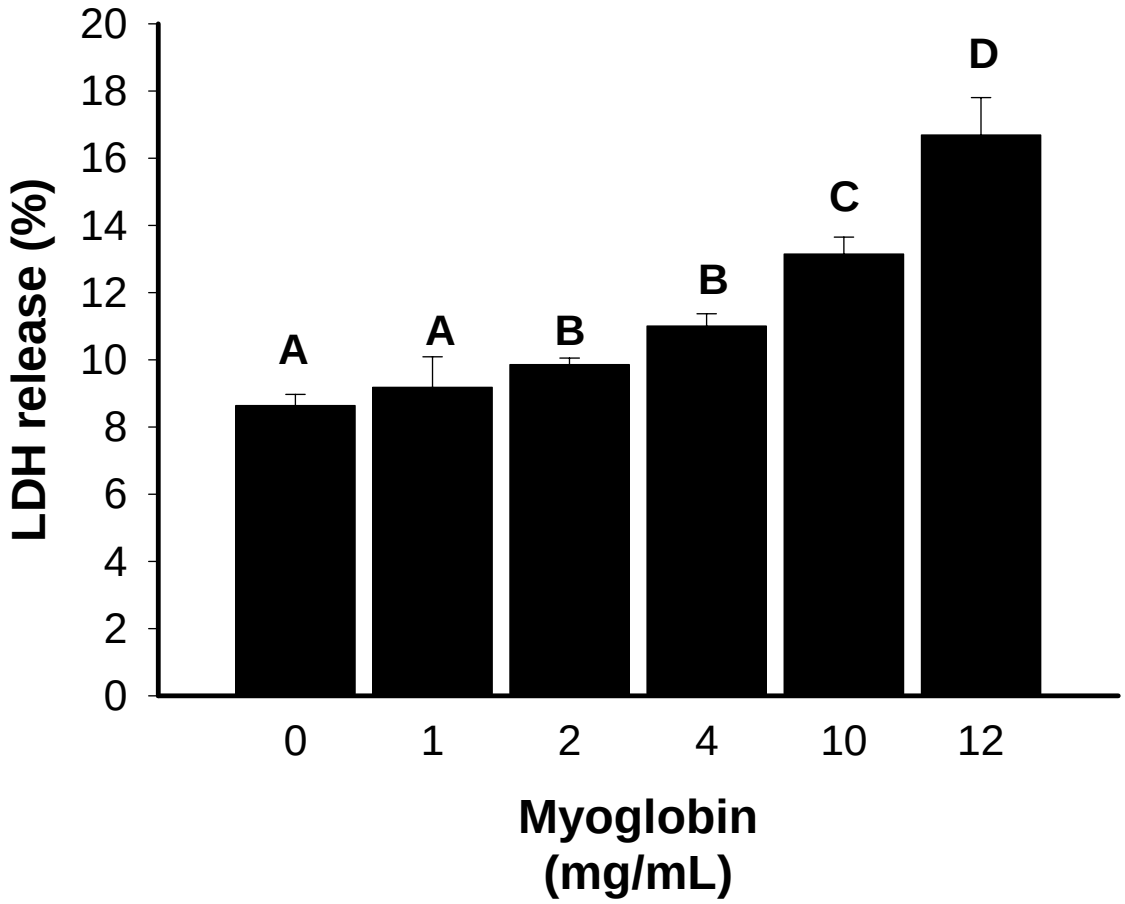


Figure 1. LDH release in response to various concentrations of myoglobin

Renal slices were incubated with myoglobin (0-12 mg/mL pretreated with 4 mM ascorbic acid) for 120 minutes at 37°C. LDH release data are expressed as percent of total LDH. Values represent mean ± SEM with n=4 animals. Concentrations with dissimilar superscripts are statistically ($p<0.05$) different from one another.

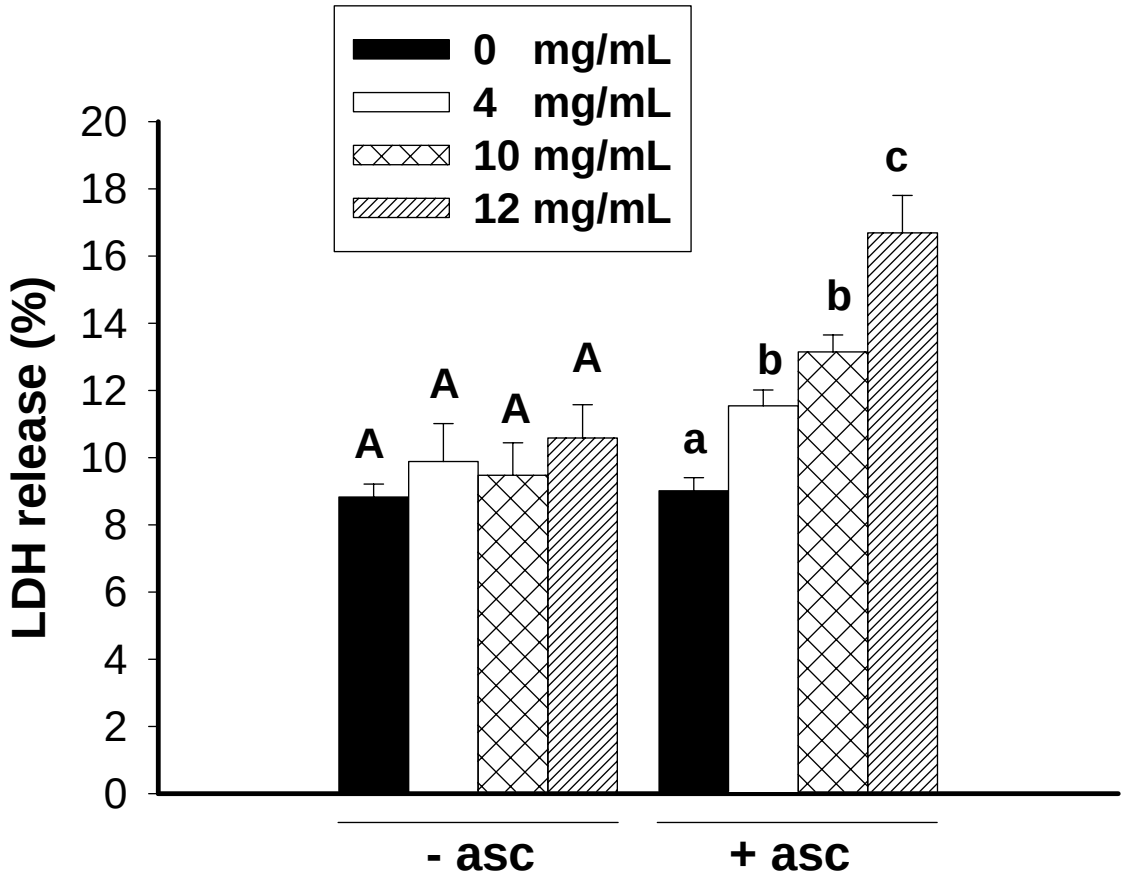


Figure 2. LDH release in response to ascorbate pretreatment of myoglobin

Renal slices were incubated with myoglobin (0-12 mg/mL pretreated with 0 mM or 4 mM ascorbic acid (asc)) for 120 minutes at 37°C. LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

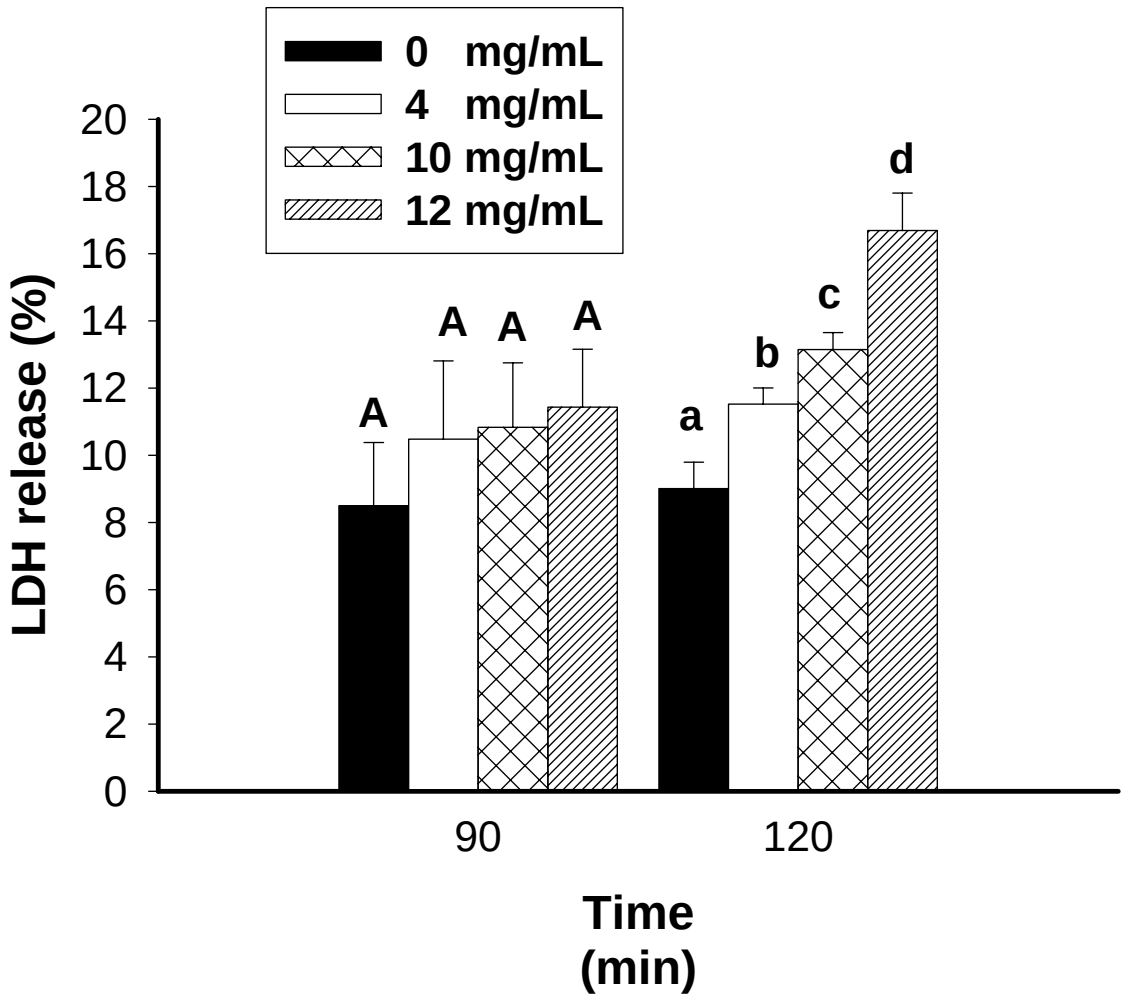


Figure 3. Time course of myoglobin-induced LDH release

Renal slices were incubated with myoglobin (0-12 mg/mL) for 90 or 120 minutes at 37°C. LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each incubation period.

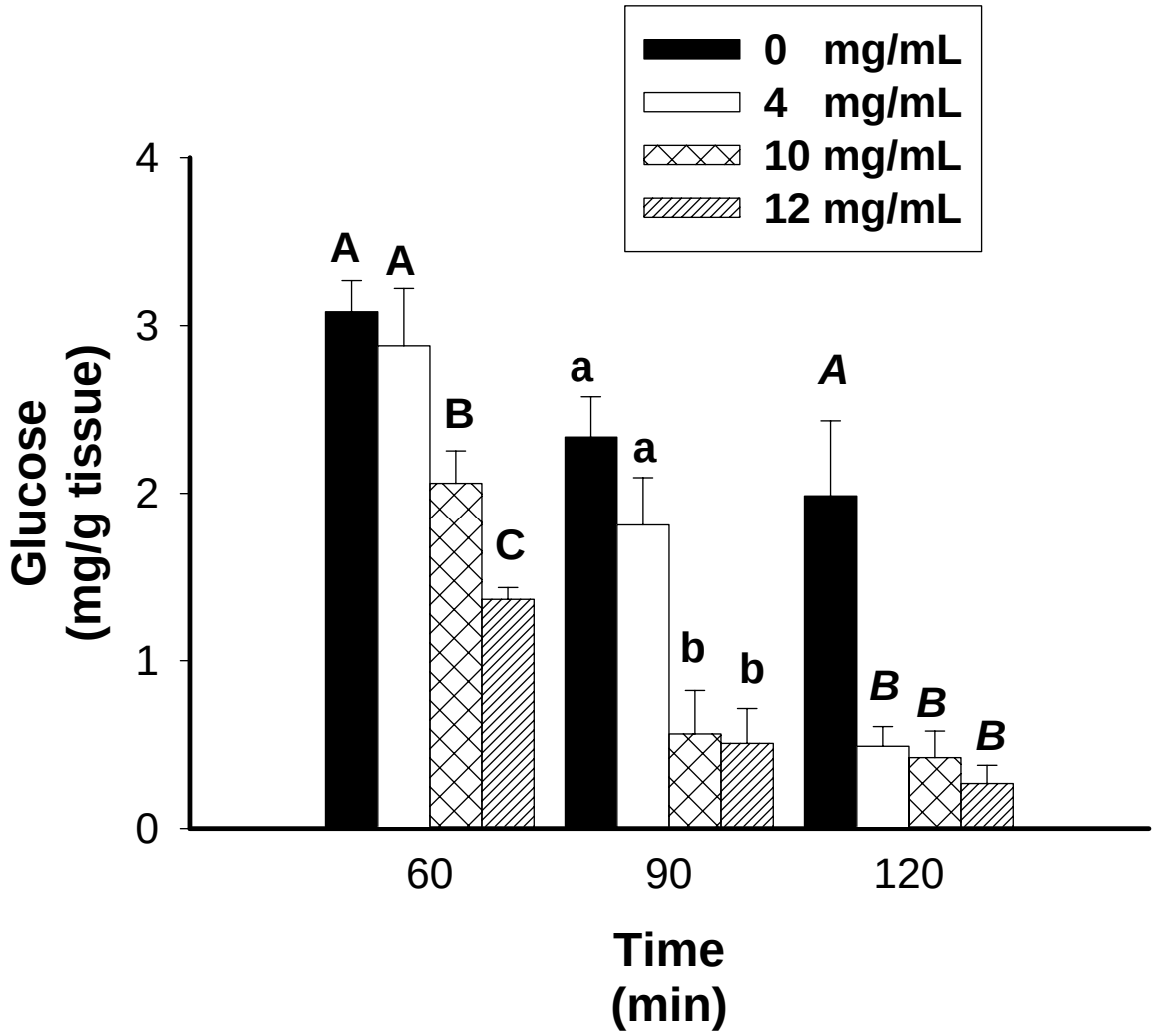


Figure 4. Time course of myoglobin-induced loss of gluconeogenesis

Renal slices were incubated with myoglobin (0-12 mg/mL) for 60-120 minutes at 37°C. To stimulate gluconeogenesis, pyruvate (10 mM final) was added during the last 30 minutes of incubation. Gluconeogenesis data are expressed as amount glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each incubation period.

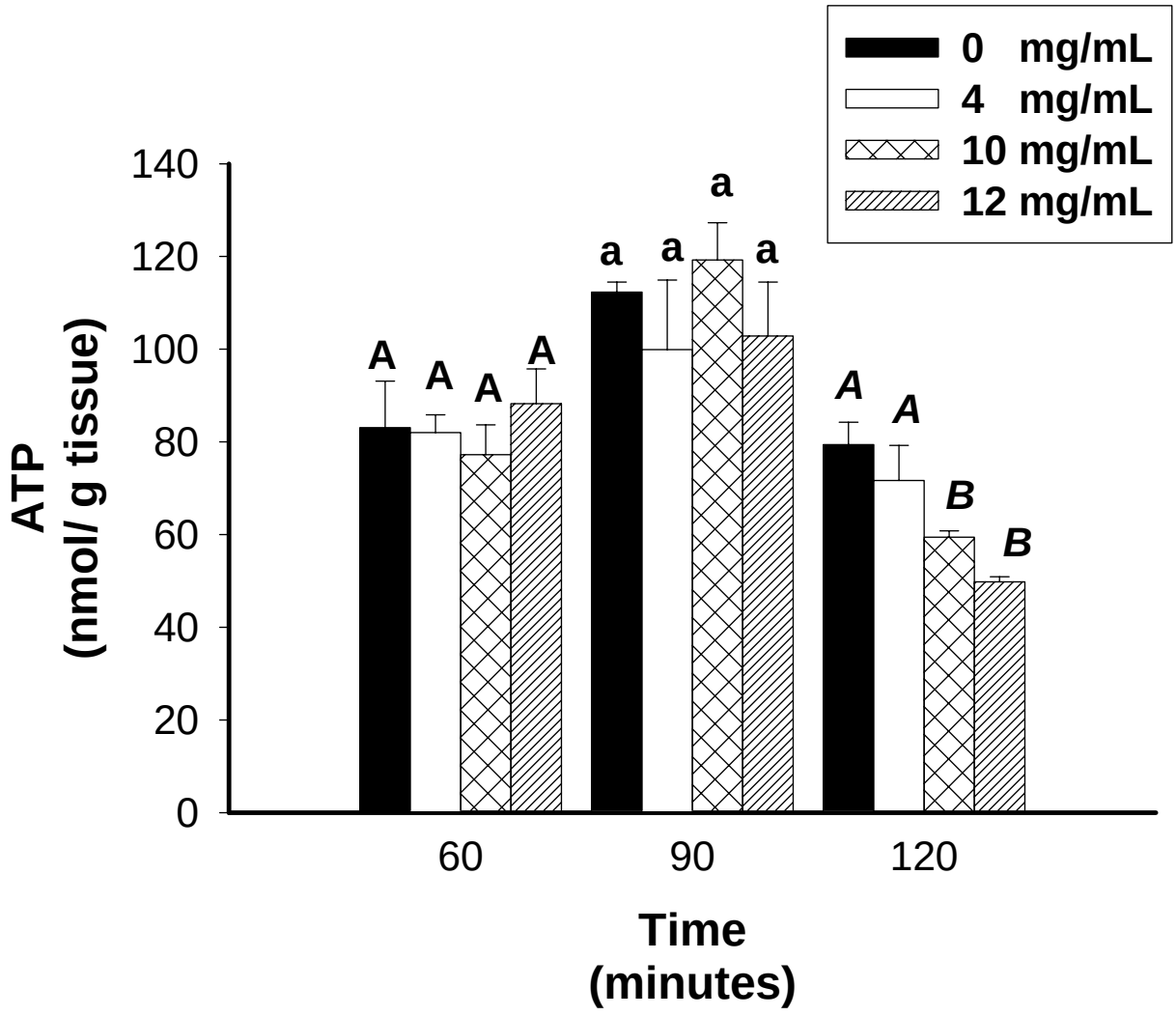


Figure 5. Time course of myoglobin-induced alterations in ATP levels

Renal slices were incubated with myoglobin (0-12 mg/mL) for 60-120 minutes at 37°C. Samples were extracted and analyzed for ATP. Data are expressed as amount ATP (nmol) per gram (g) tissue. Values represent mean ± SEM with n=4-6 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each incubation time.

Table 1. Time course of myoglobin-induced lipid peroxidation

Incubation (min)	Myoglobin (mg/mL) 0	4	10	12
	nmol/g tissue	nmol/g tissue	nmol/g tissue	nmol/g tissue
60	75.73 ± 5.38^{A}	110.48 ± 18.09^{B}	98.56 ± 15.57^{B}	115.41 ± 20.59^{B}
90	60.30 ± 1.94^{A}	81.07 ± 5.07^{B}	91.01 ± 1.95^{B}	84.21 ± 1.03^{B}
120	114.20 ± 3.76^{A}	145.23 ± 9.09^{B}	135.60 ± 3.92^{B}	140.50 ± 6.34^{B}

Renal slices were incubated with myoglobin (0-12 mg/mL) for 60-120 minutes at 37°C. Lipid peroxidation was measured as described in methods. Data are expressed as amount MDA (nmoles) per gram (g) tissue. Values represent mean ± SEM with n=4-7 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each incubation period.

Table 2. Time course of myoglobin-induced changes in glutathione levels

	Incubation (min)	Myoglobin (mg/mL) 0	4	10	12
Total (nmol/mg tissue)	60	0.635 ± 0.042^{A}	0.544 ± 0.029^{B}	0.498 ± 0.022^{B}	0.490 ± 0.035^{B}
	90	0.528 ± 0.052^{A}	0.361 ± 0.038^{B}	0.319 ± 0.022^{B}	0.286 ± 0.015^{B}
	120	0.486 ± 0.035^{A}	0.422 ± 0.033^{A}	0.358 ± 0.018^{B}	0.308 ± 0.028^{B}
GSSG (%GSSG/total)	60	8.956 ± 1.192^{A}	7.969 ± 0.882^{A}	7.651 ± 1.153^{A}	7.069 ± 1.102^{A}
	90	8.768 ± 0.484^{A}	10.557 ± 0.336^{B}	10.084 ± 0.396^{B}	10.604 ± 0.425^{B}
	120	7.079 ± 0.755^{A}	10.209 ± 0.942^{A}	11.653 ± 1.085^{B}	16.020 ± 1.677^{C}

Renal slices were incubated with myoglobin (0-12 mg/mL) for 60-120 minutes at 37°C. Samples were extracted and analyzed for glutathione content as described in methods. Total glutathione levels are expressed as nmoles total per (mg) tissue. Glutathione disulfide (GSSG) levels are expressed as %GSSG of total glutathione. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each incubation period.

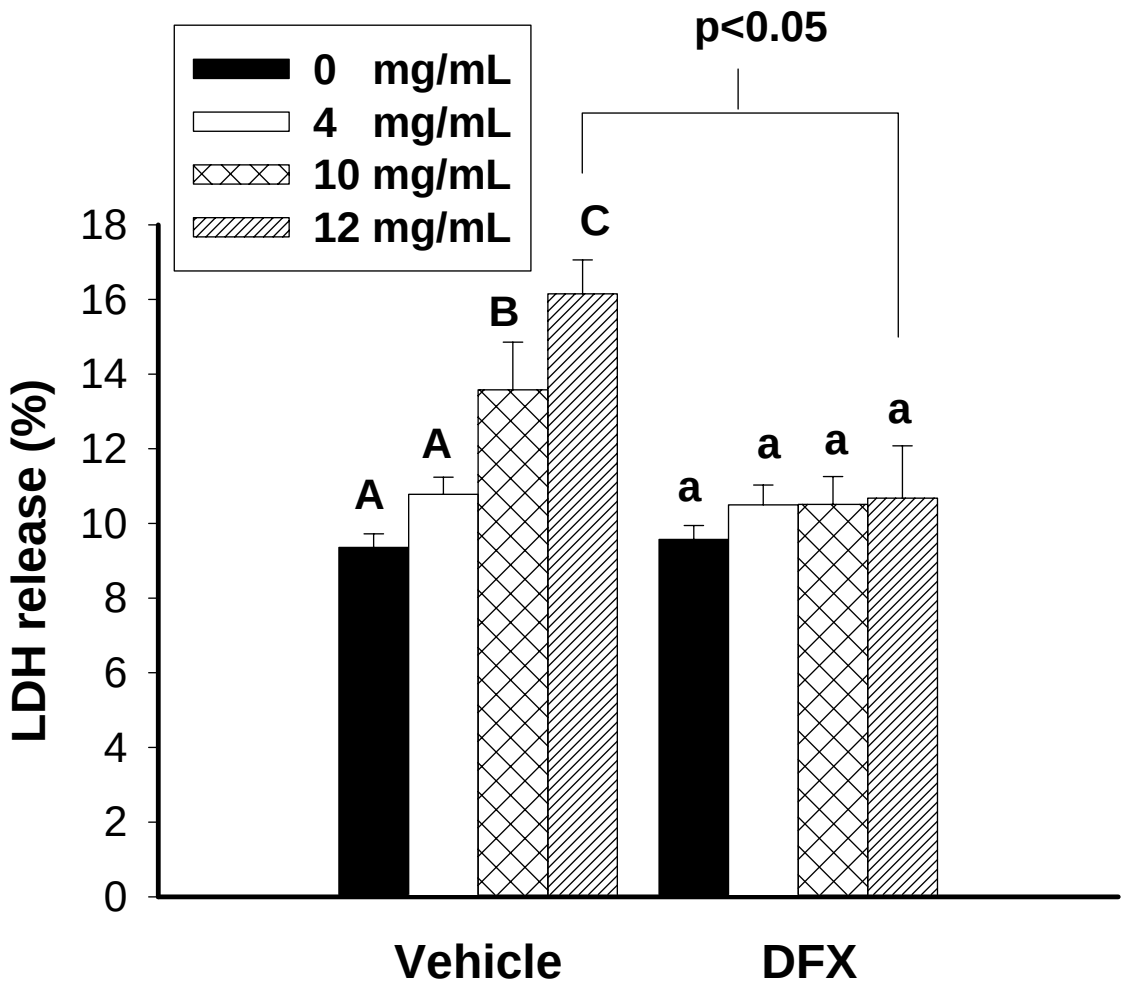


Figure 6. Effect of DFX on myoglobin-induced LDH release

Renal slices were pretreated with 0.1mM DFX for 15 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

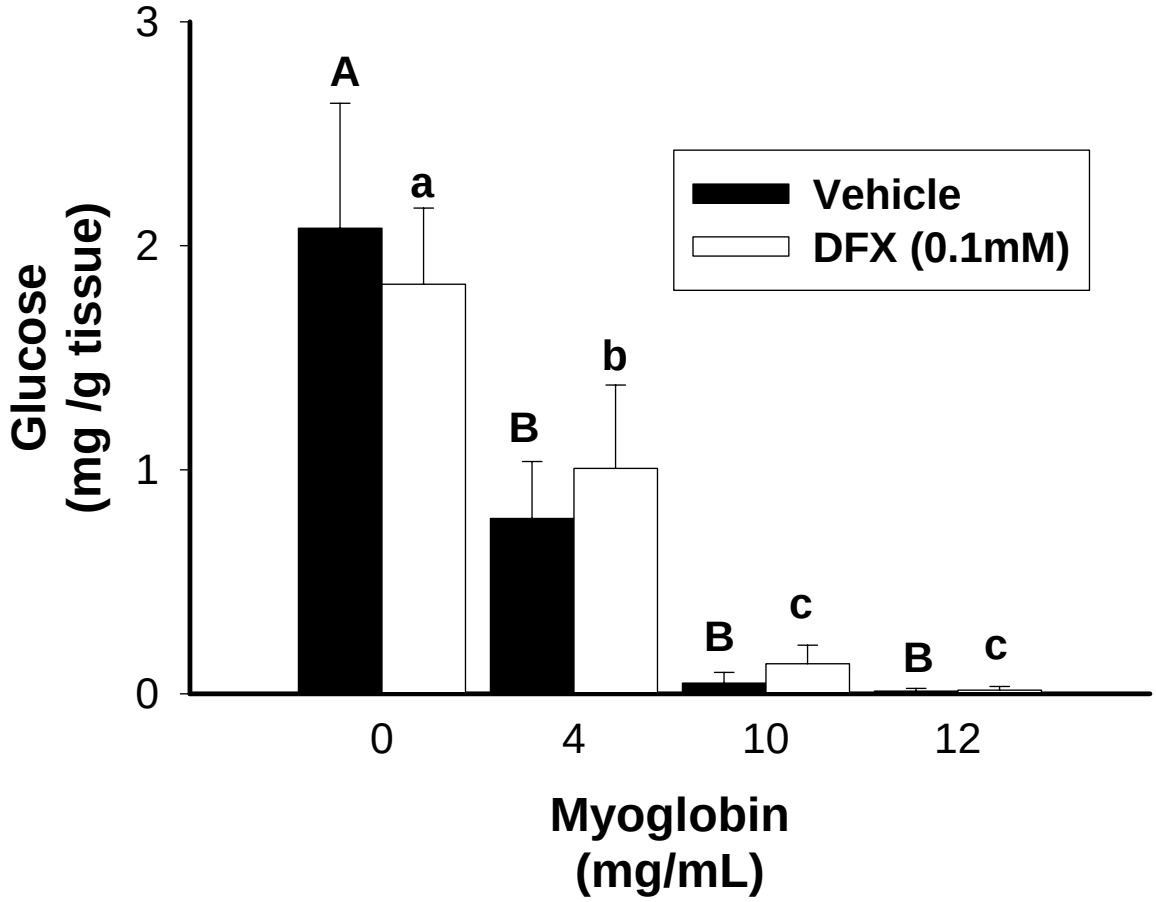


Figure 7. Effect of DFX on myoglobin-induced loss of gluconeogenesis

Renal slices were pretreated with 0.1mM DFX for 15 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). To stimulate gluconeogenesis, pyruvate (10 mM final) was added during the last 30 minutes of incubation. Gluconeogenesis data are expressed as glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

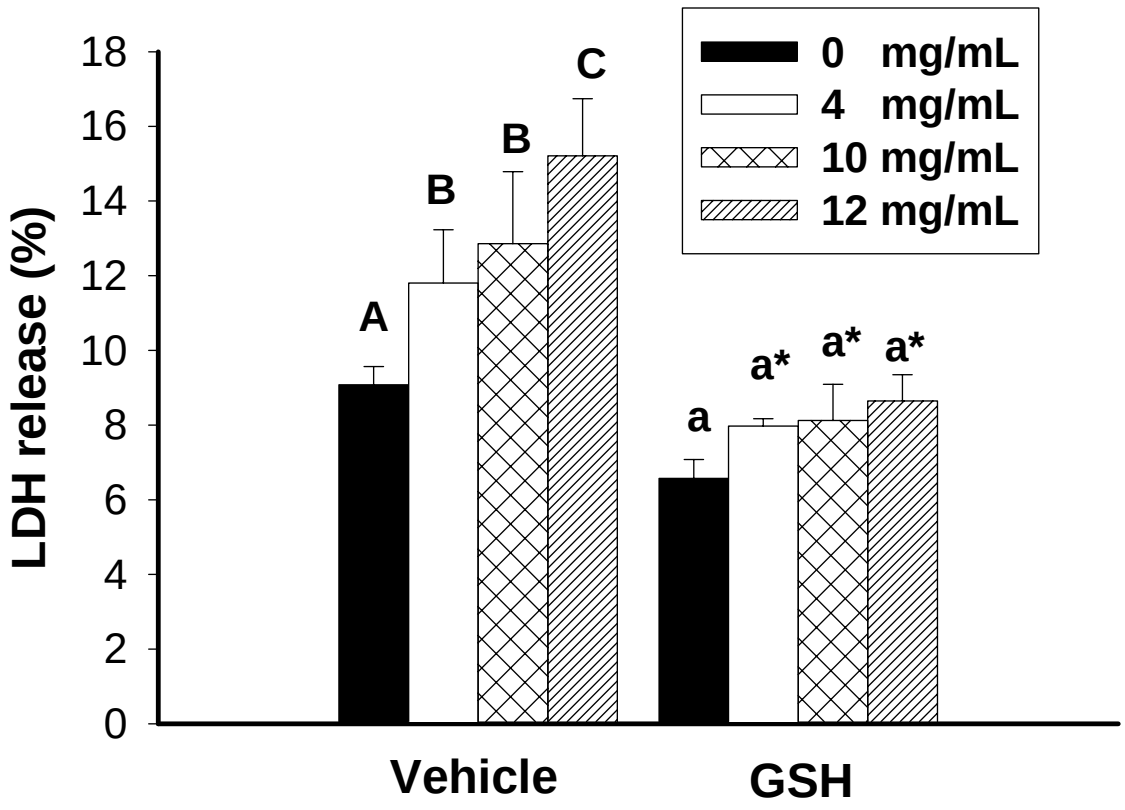


Figure 8. Effect of GSH on myoglobin-induced LDH release

Renal slices were pretreated with 1mM glutathione for 30 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each pretreatment. An asterisk (*) indicates statistical ($p<0.05$) difference between pretreatments within corresponding concentrations of myoglobin.

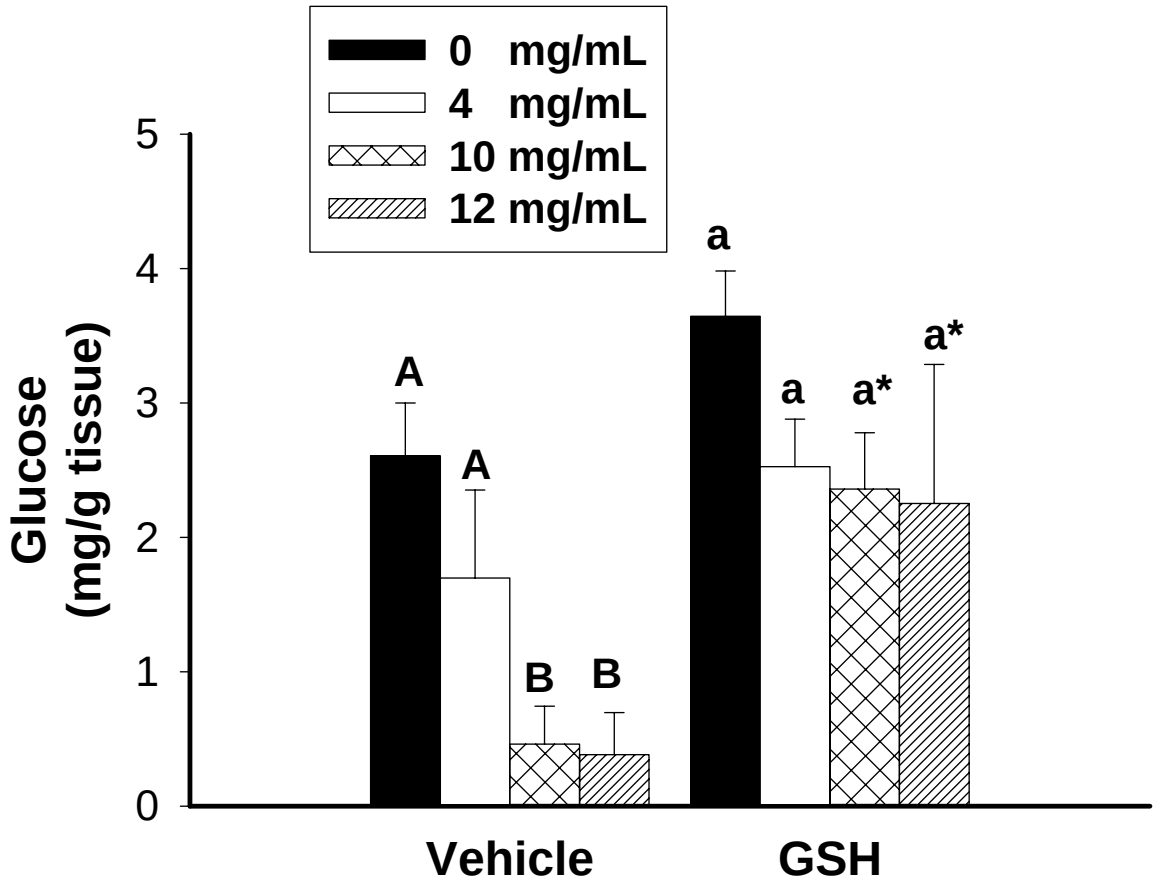


Figure 9. Effect of GSH on myoglobin-induced loss of gluconeogenesis

Renal slices were pretreated with 1mM glutathione for 30 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). To stimulate gluconeogenesis, pyruvate (10 mM final) was added during the last 30 minutes of incubation. Gluconeogenesis data are expressed as glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each pretreatment. An asterisk (*) indicates statistical ($p<0.05$) difference between treatments, within corresponding concentrations of myoglobin.

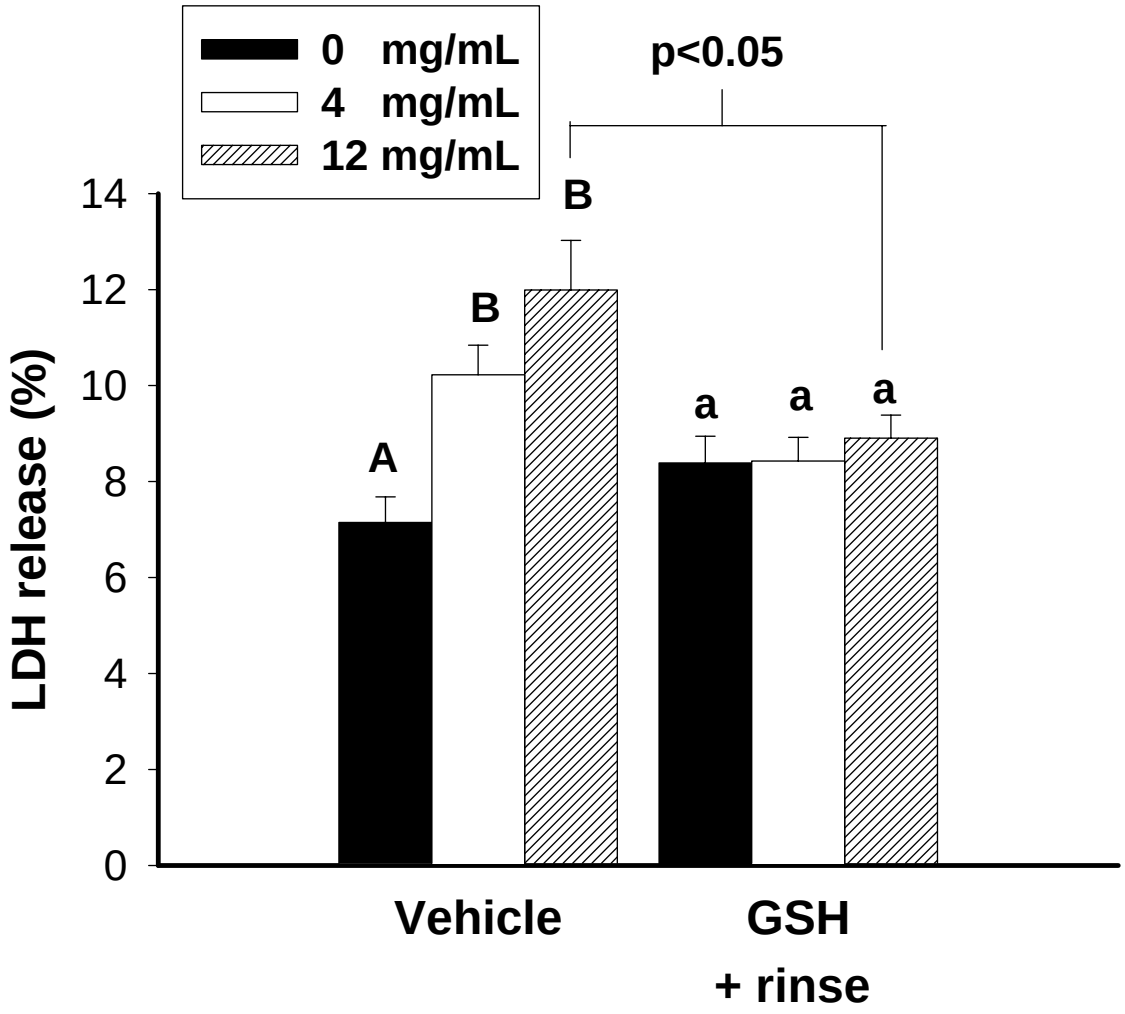


Figure 10. Effect of GSH+ rinse on myoglobin-induced LDH release

Renal slices were pretreated with 1mM glutathione for 30 minutes at 37°C. Following pretreatment, the tissues were placed in pre-warmed Krebs-Ringer buffer (lacking GSH) and incubated an additional 90 minutes with myoglobin (0-12 mg/mL). LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

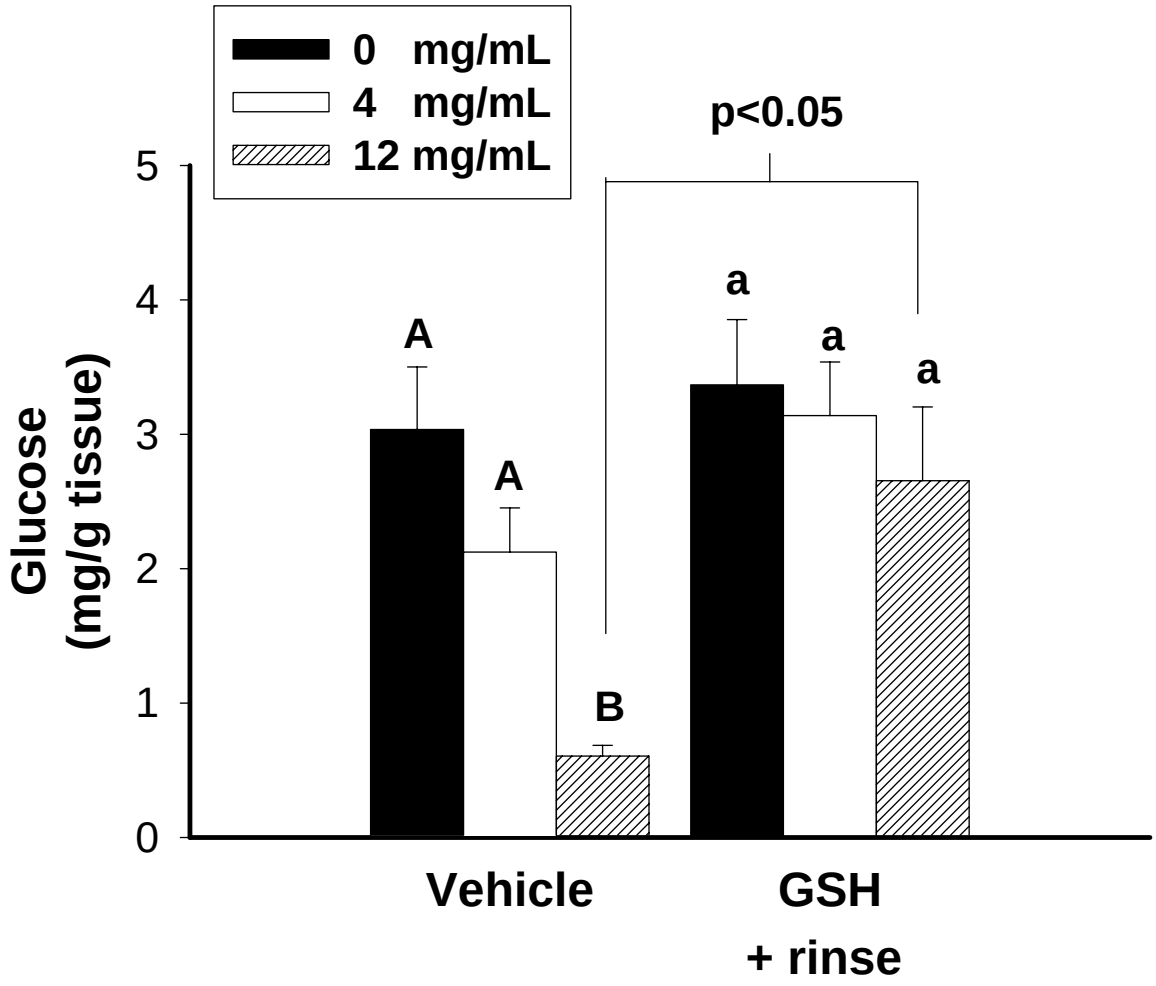


Figure 11. Effect of GSH + rinse on myoglobin-induced loss of gluconeogenesis

Renal slices were pretreated with 1mM glutathione for 30 minutes at 37°C. Following pretreatment, the tissues were placed in pre-warmed Krebs-Ringer buffer (lacking GSH) and incubated an additional 90 minutes with myoglobin (0-12 mg/mL). To stimulate gluconeogenesis, pyruvate (10 mM final) was added during the last 30 minutes of incubation. Gluconeogenesis data are expressed as glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

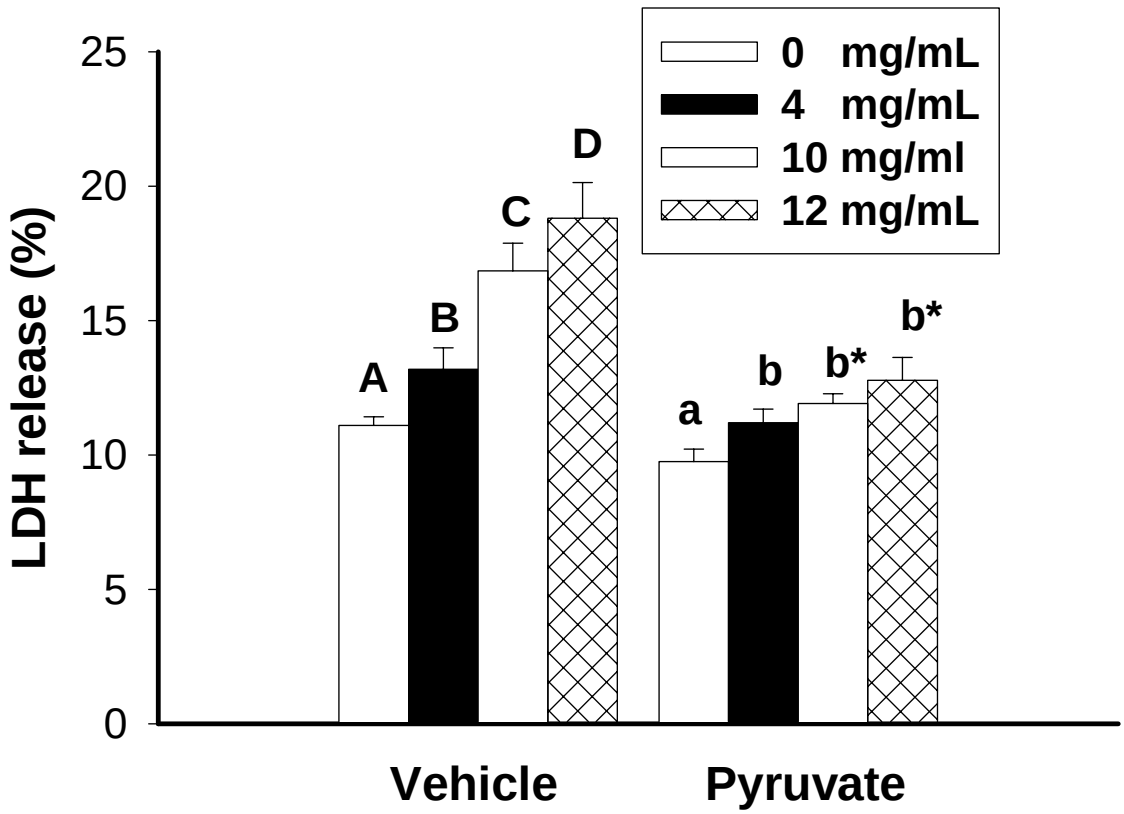


Figure 12. Effect of pyruvate on myoglobin-induced LDH release

Renal slices were incubated with pyruvate (10 mM final) and myoglobin (0-12 mg/mL) for 120 minutes at 37°C. LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment. An asterisk (*) indicates statistical ($p<0.05$) difference between treatments, within corresponding concentrations of myoglobin.

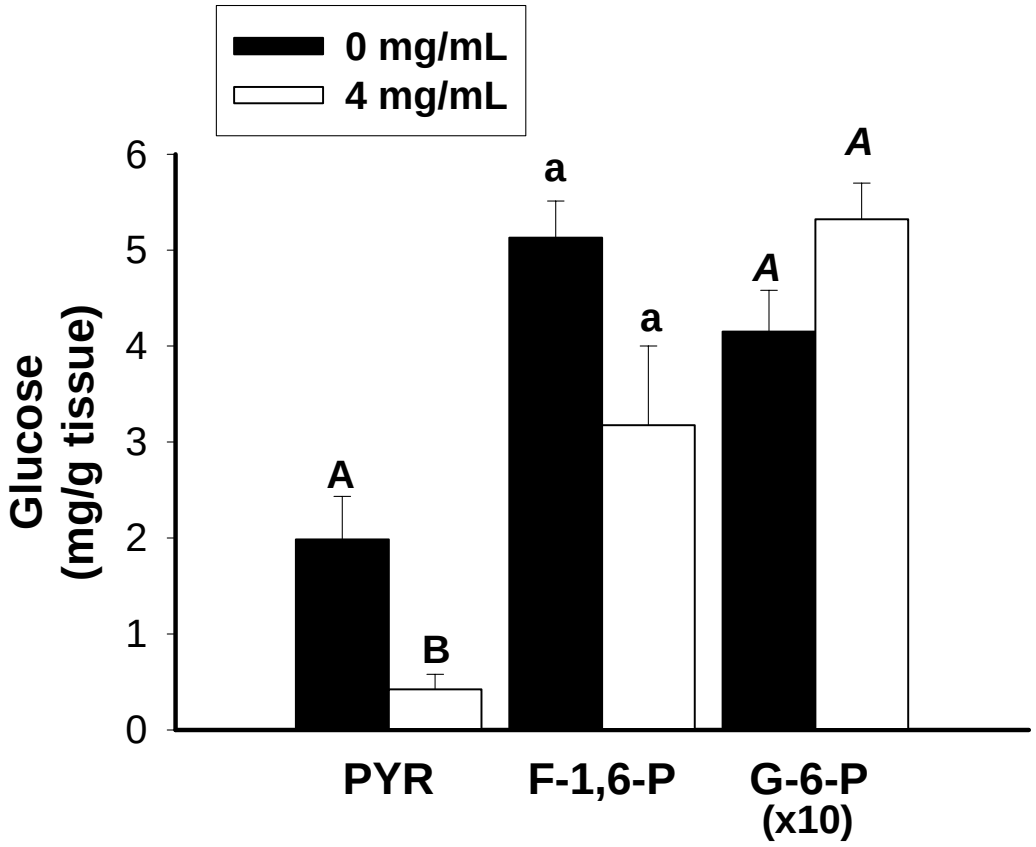


Figure 13. Stimulated gluconeogenesis with various substrates in the presence of myoglobin

Renal slices were incubated with myoglobin (0 or 4mg/mL) for 120 minutes at 37°C. Gluconeogenesis was stimulated by the addition of various substrates (10 mM final): pyruvate (Pyr), fructose-1,6-diphosphate (F-1,6-P), or glucose-6-phosphate (G-6-P) for the last 30 minutes of incubation. Gluconeogenesis data are expressed as glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each substrate.

Table 3. Microsomal assay

	Myoglobin (mg/mL)	
Microsomes	0	4
	AU/min	AU/min
+	0.0200 ± 0.00117^{A}	0.0250 ± 0.00257^{A}
-	0.0191 ± 0.00192^{A}	0.0265 ± 0.00270^{A}

Incubations were performed in the presence or absence of microsomes with 0 or 4 mg/mL myoglobin. Data are expressed as change in absorbance (AU) per minute to indicate a potential detoxification of radicals. Values represent mean ± SEM with n=4. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another.

1.4. DISCUSSION

The mechanism of myoglobin toxicity is not completely understood. The goal of this study was to characterize myoglobin toxicity in a renal cortical slice model and to develop a hypothesis to explain the basic mechanism of toxicity. The chosen slice model should serve the purpose by eliminating many confounding factors. In order to make any substantial conclusions about mechanisms, the myoglobin toxicity first must be characterized in the slice model. The experiments in this chapter were designed to address this issue in addition to providing some basic mechanistic information. Concentration-response and time-course studies, as well as protection assays, were employed. Moreover, other experiments addressed questions concerning intracellular sites of toxicity.

1.4.1 Concentration-response study

Previous *in vitro* investigations have used concentrations of 0-40 mg/mL myoglobin. Final concentrations in this study ranged from 0-12 mg/mL myoglobin. This level was well within the previous clinical and experimental studies and in keeping with the solubility of myoglobin. This concentration range, when applied to slices for two hours, caused loss of membrane integrity as measured by LDH release (Figure 1). A significant loss of membrane integrity was observed with concentrations as low as 2 mg/mL myoglobin. These findings corresponded with previous literature that reports concentrations above 4 mg/mL, when incubated with HK-2 cells, were lethal. In one study, 1 mg/mL of myoglobin produced no death, only growth arrest (Iwata, 1996). This suggests that the renal slice model is at least as sensitive as the HK-2 cell model; or

possibly more sensitive based on the fact the myoglobin as low as 2 mg/mL caused LDH release. Because concentrations of 0, 4, 10, and 12 mg/mL myoglobin demonstrated the best concentration-dependent effect, these concentrations were chosen as the test concentrations for the remainder of the study.

The myoglobin used in this study was predominately the metmyoglobin form (Fe^{3+}) as purchased. Ascorbic acid was used for the reduction of the iron to the ferrous form. Pretreatment of the metmyoglobin with 0 or 4 mM ascorbic acid greatly affected the toxicity. LDH release failed to rise above baseline if the myoglobin was not pretreated with ascorbic acid (Figure 2). However, pretreatment with ascorbic acid, once again produced a concentration-dependent increase in the LDH release. Other investigators (Zager and Foerder, 1992; Zager *et al.,* 1997) reported that myoglobin must be pretreated with ascorbic acid to induce toxicity. This indicated that incubation with the ferrous form (Fe^{2+}) of the myoglobin was required and thus, pretreatment of myoglobin with ascorbic acid must be done to ensure the myoglobin is in the reduced form when presented to the tissues. For the remainder of the study myoglobin was pretreated with 4 mM ascorbic acid.

Taken as a whole, the concentration study also indirectly answered a question concerning the basic mechanism of toxicity. Prior evidence suggested that intraluminal mechanisms due to the increased concentrations of myoglobin in the urine were responsible for myoglobin toxicity (Clyne *et al.*, 1979). The slice model provided an ideal physiological environment to address this question. Because, the tubules in the slice model were collapsed, any toxicity demonstrated would not be dependent on

intraluminal events. Therefore, these experiments indicated intraluminal events were not necessary to induce toxicity.

1.4.2 Time-course study

The concentration-response study was done at an arbitrarily chosen incubation time period. Because of the relatively short lifespan of the renal slices, two hours was estimated to be a sufficient incubation time to realize any detectable toxicity. In order to evaluate a time frame for myoglobin toxicity, incubation times other than two hours were performed. LDH release was used to designate loss of viability. At two hours incubation, as previously shown in Figure 1, a significant concentration-dependent increase in LDH release was observed. The time frame corresponds with the *in vivo* time frame reported by Zager *et al.* (1995). Tubules were harvested four hours post-glycerol or sham injection, at a time when urine myoglobin levels approximated 40 mg/mL. These tubules were incubated for 90 minutes to determine the rate of *in vitro* expression of the toxicity as measured by LDH release. At 75 minutes, LDH release in the glycerol treated samples was approximately 30% whereas the controls were about 10%. Although this was earlier than two hours, the difference may be attributed to the significantly greater concentration of myoglobin. However, the difference may also be due to the hemodynamic effects and precipitation of myoglobin within the lumen. In addition, the release of phosphates, calcium ions, and organic acids with the glycerol model could modify the results.

In addition to LDH release, gluconeogenesis was chosen as a second measure of toxicity for evaluating the basic time frame. When slices were treated with myoglobin,

a significant, concentration-dependent loss of gluconeogenesis, as measured by the amount of glucose per gram tissue, was observed as early as one hour of incubation (Figure 4). Notably, this loss of cell function was one hour prior to loss of viability. As incubation times increased, gluconeogenesis was essentially abolished. Myoglobin did not induce a decline in gluconeogenesis by interfering with the glucose assay as myoglobin shifted the glucose curves with respect to concentration (Figure 5). This observation indicated that myoglobin was not interacting with the reagents of the assay and therefore, could be subtracted as background. Moreover, as further evidence, the decline of levels of gluconeogenesis was more pronounced in the presence of myoglobin (Figure 4). Taken as a whole, the time-course study revealed loss of cell function occurring as early as one hour while significant loss of viability did not occur until two hours. This outlined a basic time frame from which to work with loss of gluconeogenesis occurring much earlier than LDH release.

The remaining three measures of toxicity, namely ATP levels, lipid peroxidation, and glutathione levels, were employed to investigate further the time frame and possible associated events of toxicity. Because gluconeogenesis was affected, myoglobin could have been acting to decrease the energy as noted by ATP levels. Examination of ATP levels would address this possibility and answer a more general question– what happens to nucleotide levels during myoglobin toxicity? Previous literature has reported varying results concerning alterations in ATP levels. Trifillis *et al.* (1981) showed a decline in ATP content in renal cortex of rats with glycerol-induced rhabdomyolysis. Zager (1996a) also reported a drop in ATP levels in proximal tubules segments isolated from glycerol-injected mice, but concluded that the decline was due to LDH release and

loss of viability. However, a slight change in the ATP/ADP ratio was noted. In this study, when slices were incubated with myoglobin, ATP levels remained unchanged from control at 60 and 90 minutes of incubation (Figure 5). Conversely, at two hours, the levels significantly declined with a concentration-dependent trend. This time frame coincided with significant loss of viability. In consideration of the relationship between LDH release and ATP changes, the decline in ATP levels seemed to have been caused by cell death. Two pieces of evidence supported this conclusion. The percentage of LDH release above control in the myoglobin-treated group was the same percentage of decline below control in ATP levels. Lastly, as detailed in Chapter 2, when renal slices were incubated with pyruvate, the percent of LDH release over control with the 12 mg/mL myoglobin treatment was ~30%, as was the percentage of ATP decline in the 12 mg/mL myoglobin treated versus control.

In opposition to ATP levels, lipid peroxidation and changes in glutathione levels occurred before the loss of viability. Lipid peroxidation, as measured by malondialdehyde levels, was assessed to determine any possibility of radical involvement. The occurrence of myoglobin-induced lipid peroxidation *in vitro* and *in vivo* has been widely recounted (Shah and Walker, 1988; Moore *et al.*, 1998; Holt *et al.*, 1999). Illustration of myoglobin-induced lipid peroxidation in the slice model would further confirm the model as well as provide mechanistic and time-frame information. Slices incubated with myoglobin for one hour manifested a significant elevation in lipid peroxidation over controls (Table 1). Similar to gluconeogenesis, the effect was one hour prior to loss of viability. This sequence of events corresponded to those reported by Zager *et al.* (1995). As outlined before, tubules were harvested four hours post-

glycerol or sham injection, at a time when urine myoglobin levels approximated 40 mg/mL. These tubules were incubated for 90 minutes to determine the rate of *in vitro* expression of the toxicity as measured by LDH release. A significant increase in MDA levels at time 0 (of a 90 minute incubation) reportedly preceded a rise in LDH release occurring at 75 minutes in the glycerol-treated group. Taken together, these data suggest lipid peroxidation could be a mediator in loss of viability given that it occurred well in advance of LDH release. Furthermore, this implicated free radical involvement as a component of the toxicity.

Like lipid peroxidation, changes in glutathione levels occurred by one hour. Because lipid peroxidation was first evident at one hour, changes in glutathione levels were evaluated to confirm the involvement of free radicals. Slices treated for one hour with myoglobin showed a significant decrease from control in the total glutathione levels, as expressed as nmol per gram tissue (Table 2). If the reduction was due to detoxification of radicals, then theoretically, the percentage of glutathione disulfide levels should have increased. The amount of glutathione disulfide (GSSG), as measured by %GSSG of total glutathione, did not significantly increase until 90 minutes (Table 2). However, due to the low detection limit of the GSSG, it was possible that the levels actually increased earlier. Overall, the changes in glutathione levels, total and GSSG, validated the lipid peroxidation at one hour and confirmed the involvement of radicals in myoglobin toxicity. Moreover, these findings agreed with previous findings concerning depletion of glutathione in the glycerol model of myoglobin toxicity. Abul-Ezz *et al.* (1991) reported that injection of glycerol into rats produced a significant and early depletion of glutathione. In this study, the time study of glutathione levels and lipid

peroxidation indicated free radical generation events preceded loss of viability; and thus, may have been causally linked to the ultimate demise of the tubules.

Taken together, the basic measures of toxicity established a time course for the events of myoglobin toxicity. Gluconeogenesis, lipid peroxidation, and changes in glutathione levels were detected as early as one hour whereas LDH release and changes in ATP levels were detected at two hours. In other words, the early insult was oxidative in nature and may have played a major role in the loss of membrane integrity and decline of ATP levels one hour later.

1.4.3 Protection assays

Protection assays were utilized to further probe the time frame for clues to mechanistic events. Three protection assays are presented in this chapter. The protective effect of a thiol-containing agent (GSH), an iron chelator (DFX), and an α-ketoacid (pyruvate) were chosen for various reasons. Because changes in glutathione levels and thus possibly free radicals are implicated in the mechanism of toxicity, the application of exogenous reduced glutathione (GSH) was used as an attempt to inhibit the toxicity. Slices were pretreated with GSH for 30 minutes prior to a two-hour incubation with myoglobin. The pretreatment attenuated myoglobin toxicity as measured by LDH release (Figure 8) and gluconeogenesis (Figure 9). GSH produced a significant reduction in the LDH release and a significant increase in gluconeogenesis at all concentrations of myoglobin. The protection of gluconeogenesis not only alluded to the involvement of a radical, but when taken with the DFX effect on gluconeogenesis

(Figure 7), implied that this part of the mechanism of toxicity is oxidative and iron-independent.

To exclude any possibility of a media interaction of GSH with myoglobin and thus detoxification, the protection assay was repeated with a rinse between the GSH and myoglobin incubations. Slices were pretreated for 30 minutes with GSH. Prior to a two-hour incubation with myoglobin, the slices were placed into fresh pre-warmed buffer. GSH continued to provide protection against loss of viability with only a 30-minute loading period (Figure 10). Moreover, GSH maintained protection of gluconeogenesis (Figure 11). These results indicated that the protection mediated by GSH was not due to an extracellular interaction and thus possible detoxification of myoglobin in the media. Furthermore, slices loaded with GSH for only 30 minutes, were capable of maintaining viability.

Previous literature agreed that deferoxamine (DFX) could guard against myoglobin toxicity (Paller, 1988; Zager and Burkhart, 1997). This finding was the only one that was consistent among all the experimental models. Therefore, this study sought to investigate the protective effect of DFX to verify the slice model as a relevant model for studying myoglobin toxicity. Furthermore, the use of DFX would allow investigation of individual toxic events along the constructed time line. Slices were pretreated for 15 minutes with DFX prior to a two-hour incubation with myoglobin. LDH release in the presence of DFX treatment was not significantly different in the myoglobin group as compared to control. In addition, at the high concentration of myoglobin, DFX-pretreated tissue had less LDH release than vehicle treated slices. On the contrary, DFX pretreatment did not provide protection against myoglobin-induced changes in

gluconeogenesis. In fact, the fall in glucose levels was indistinguishable between DFX and DFX-vehicle pretreated tissues exposed to myoglobin. The effects of DFX suggested several conclusions concerning gluconeogenesis and myoglobin toxicity. For one, the loss of gluconeogenesis was iron-independent. This also suggested a lack of participation of hydroxyl radicals because their formation is propagated via Fenton reactions which involve iron. Moreover, because DFX can detoxify ferryl myoglobin radicals, the results suggested that the effect on gluconeogenesis was not via the ferryl radical. It is possible that oxygen radicals, produced in the mitochondria, could have been causing the compromise of gluconeogenesis in addition to initiating oxidative events. Zager (1996a) implicated the terminal mitochondrial transport chain as the origin of free radicals that initiate injury in myoglobin toxicity. Also, because there was dramatic loss of gluconeogenesis with retention of membrane integrity, loss of gluconeogenesis probably was not a major contributing factor to loss of viability. In general, the effect of DFX on LDH release and the lack of DFX protection on myoglobin-induced changes in gluconeogenesis indicated that the mechanism of myoglobin toxicity was iron-dependent, but that not every aspect of the toxicity was iron-dependent.

The protective effect of pyruvate was investigated for two basic reasons. First, because pyruvate was used to stimulate gluconeogenesis, it was possible that pyruvate affected toxicity. In addition, pyruvate can detoxify radical pathways by scavenging H_2O_2 and previous literature consistently reports that H_2O_2 levels are elevated in myoglobin toxicity. Co-incubation of the slices with pyruvate and myoglobin for two hours revealed that pyruvate, indeed, was affecting toxicity. Myoglobin-induced LDH release was significantly reduced in the pyruvate-treated groups as compared to

vehicle-treated group (Figure 12). At the 10 and 12 mg/mL concentrations of myoglobin, LDH release was significantly lower in the pyruvate-treated group than in the vehicle-treated group. Because of the free radical implications, pyruvate was further addressed in detail in this study and the results of which are reported in Chapter 2.

1.4.4 Intracellular targets

Previous studies have investigated potential targets of myoglobin toxicity. Previous literature reports that myoglobin induces necrotic cell death as opposed to apoptosis. One study indicated that the nucleus was not a target as indicated by a lack of TUNNEL positive proximal tubule cells, no structural evidence of apoptosis, no oxidative damage to DNA, and no DNA laddering (Nath *et al.*, 1998). However, several investigators reported that the mitochondria were targets for myoglobin (Nath *et al.*, 1998, 100; Zager, 1996a). This study sought to investigate if effects of toxicity could be localized to the cytosol, mitochondria, or both. Gluconeogenesis begins in the mitochondria and continues in the cytosol. By possessing irreversible steps in each compartment, gluconeogenesis becomes a tool for answering the nature of targets of myoglobin toxicity. To better ascertain the effect of myoglobin on gluconeogenesis, three different substrates were used, two cytosolic (fructose-1,6-diphosphate and glucose-6-phosphate) and one mitochondrial (pyruvate). The low concentration of myoglobin was chosen as the test concentration because of the desire to detect subtle changes. If the higher concentrations were chosen, the toxicity may have overwhelmed cellular function and made the results difficult to interpret. When gluconeogenesis was stimulated with pyruvate, the myoglobin toxicity was evident by the decrease in the

amount of glucose per gram tissue (Figure 13). However, when fructose-1,6-diphosphate or glucose-6-phosphate was used to stimulate gluconeogenesis in the presence of zero or 4mg/mL myoglobin, there was no difference. Furthermore, stimulation with oxaloacetate, the first cytosolic substrate in the pathway, was not affected by myoglobin. Because these cytosolic steps of gluconeogenesis were not affected by myoglobin, the site of toxicity appeared to be in the mitochondria, thus implicating mitochondria as a target.

1.4.5 Spontaneous radical formation

The previous data illustrate the involvement of free radicals in myoglobin toxicity. The question then becomes, regardless of what type of radical, was the radical spontaneously or enzymatically formed from the myoglobin? To address this, microsomal assays were performed to determine if myoglobin could induce a change in absorbance at 549 nm to signify oxidation of NADPH to NADP thus indicating detoxification of a radical. The assay was done in the presence and absence of microsomes to illustrate if any perceived effect was spontaneous or enzymatic. Under all conditions, there was no significant effect observed (Table 3), suggesting that in the presence of myoglobin alone, free radicals were not spontaneously or enzymatically (microsomal) generated.

In summary, myoglobin toxicity occurred in the renal cortical slice model. Early toxic events were lipid peroxidation and diminished glutathione levels. Late events involved loss of membrane integrity and a decline in ATP levels. The toxicity appeared to be localized in the mitochondria and to involve oxidative damage. Pretreatment with

DFX indicated that the toxicity has both an iron-dependent and iron-independent component.

CHAPTER 2. PYRUVATE PROTECTION

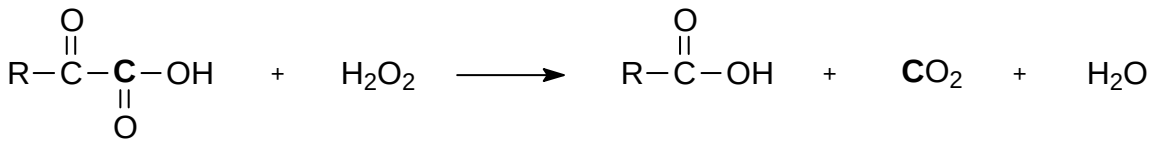
2.1. INTRODUCTION

One of the classic findings concerning myoglobin nephrotoxicity is the elevated level of H_2O_2 in tissue. Increased levels of H_2O_2 have been seen repeatedly in the glycerol *in vivo* model (Guidet and Shah, 1989; Paller, 1988). In fact, the glycerol model has been used to study various aspects of H_2O_2 toxicity (Salahudeen *et al.*, 1991). Zager (1993) further reported that myoglobin loaded proximal tubular segments, when combined with exogenous H_2O_2, exhibit increased injury. Zager and Burkhart (1997) also reported that catalase could protect proximal tubular cells exposed directly to myoglobin. These findings support the involvement H_2O_2 in myoglobin toxicity.

H_2O_2 could be contributing to myoglobin toxicity in a number of ways. Heme proteins in the presence of H_2O_2 will liberate iron (Gutteridge, 1986). Also, H_2O_2 could be participating in the initiation of free radicals by means of the Fenton reactions. Varying results have been reported for the involvement of hydroxyl radicals in myoglobin toxicity. Shah and Walker (1988) reported in the *in vivo* glycerol model using male Sprague Dawley rats, hydroxyl radical scavengers, such as dimethylthiourea (DMTU), provide marked protection against renal injury and more specifically, rises in malondialdehyde levels. However, Zager *et al.* (1995) using proximal tubular segments isolated four hours after glycerol injection into male Sprague Dawley rats reported that DMTU and benzoate did not reverse LDH release or MDA levels. In fact, using a salicylate trap method to measure the amount of hydroxyl radicals, Zager *et al*. (1995)

reported a smaller amount of hydroxyl radicals in the glycerol-injected group as compared to the vehicle-injected group. Zager and Burkhart (1997) later incubated HK-2 cells with myoglobin in the presence and absence of DMTU or benzoate. In these studies, no protective effect was observed for the hydroxyl radical scavengers against myoglobin toxicity.

The ability of DMTU to scavenge hydroxyl radicals has been well established in *in vitro* studies (Fox, 1984). Also, deferoxamine (DFX) has been shown to directly scavenge hydroxyl radicals in *in vitro* systems (Halliwell and Gutteridge, 1986), in addition to being capable of scavenging hydroxyl radicals. Actually, the protective effect of iron chelators has been taken as evidence of hydroxyl radical participation in tissue injury. This conclusion was reached because iron is critical in the generation of the hydroxyl radical in the Fenton reactions. Other chemicals can detoxify hydroxyl radicals. Simple carbohydrates have been found to have antioxidative properties (Wehmeier, 1994). In fact, glucose in physiological concentrations can act to scavenge hydroxyl radicals (Sagone *et al.*, 1983). Mannitol infusion (i.v.) is part of the treatment regimen for rhabdomyolysis. Although mannitol is a sugar, it does not act as an antioxidant, but rather as an osmotic diuretic (Zager *et al.*, 1991). α-Ketoacids can also detoxify hydroxyl radicals, albeit in an indirect way. α-Ketoacids such as pyruvate can react directly with H_2O_2 (Equation 1) (Constantopoulos and Barranger, 1984). The reaction is rapid and stoichiometric (Bunton, 1949; Melzer and Schmidt, 1988). Because pyruvate exists both in the cytosolic and mitochondrial compartments, it is capable of scavenging H_2O_2 throughout the cell.



Equation 1. Decarboxylation of α-ketoacids by H_2O_2

Pyruvate is easily transported into the cells and mitochondria (Murer and Burckhardt, 1983; Halestrap *et al.*, 1980) with intracellular concentrations of pyruvate varying among tissues. The kidney has 30 μM pyruvate and the liver has 500 μM pyruvate as an approximate concentration (Veech *et al.*, 1979; Hems and Brosnan, 1971). Previous studies have investigated pyruvate and the potential for protection. Salahudeen *et al.* (1991) incubated Sprague Dawley rat kidney homogenate with H_2O_2 (4 mM) and pyruvate (4 mM). He reported a decrease in lipid peroxidation in the samples containing pyruvate. In addition, Salahudeen reported that chromium loaded LLC-PK1 cells incubated with H_2O_2 and various concentrations of pyruvate demonstrated a decrease in concentration-dependent release of chromium, indicative of loss of viability.

Pyruvate and glucose protection was also explored in the *in vivo* glycerol model using male Sprague Dawley rats. Pyruvate (8% solution, 2.5 mL/kg) or glucose (8% solution, 2.5 mL/kg) was injected three times: just before and at 8 and 16 hours post-glycerol injection. Renal function was evaluated 8 hour following the last injection. Serum creatinine was significantly decreased in those treated with pyruvate as compared to glucose or control. Also, there was a reduction in structural injury with pyruvate treatment. It was concluded that pyruvate provided functional and structural protection (Salahudeen *et al.*, 1991). Zager and Burkhart (1998) went on to specifically

investigate pyruvate protection on HK-2 cells. LDH release reportedly was decreased in HK-2 cells co-incubated with myoglobin and pyruvate as compared to the cells incubated with myoglobin alone. Pyruvate protection also was analyzed in a different model, an ischemia-reperfusion model in cardiac tissue (DeBoer *et al.*, 1993). It was demonstrated that pyruvate protected against ATP depletion and concluded that the protection occurred through two possible mechanisms: 1) metabolic effect -possibly via action as a substrate or 2) free radical detoxification.

Previous data in Chapter 1 indicated that pyruvate did protect against myoglobin toxicity as evident by attenuation of myoglobin-induced LDH release (Figure 12). The protection afforded by pyruvate could suggest two possible mechanisms of protection. Pyruvate could be detoxifying a free radical or it could be providing a substrate for energy production. As an attempt to address the first of the two possibilities, lipid peroxidation and glutathione levels were measured after one and two hours of incubation in the presence of pyruvate. In addition, DMTU was used to simulate protection by chelation of hydroxyl radicals. The use of DMTU also would indicate any involvement of hydroxyl radicals in the mechanism of toxicity. In order to address the energy substrate possibility, ATP levels were measured to indicate any protection against the decline of ATP levels. In addition, the toxicity in the presence of high and low concentrations of glucose was compared to that in the presence of pyruvate. Together, the results would help delineate the mechanism for protection afforded by pyruvate.

2.2. MATERIALS AND METHODS

2.2.1 Chemicals and sources

(Company and catalog number)

Dimethylthiourea (Aldrich, D18870-0)

2.2.2 Incubation of renal slices.

Because pyruvate was used to stimulate gluconeogenesis, studies addressed the possibility of any influence on myoglobin toxicity. Renal slices (50-100 mg) were co-incubated with pyruvate (10 mM final concentration) and myoglobin (final concentration 0, 4, 10 or 12 mg/mL) for two hours at 37^{o}C under 100% oxygen and constant shaking (100 cycles/minute) in a Dubnoff metabolic incubator. Upon completion of the incubations period, samples were analyzed for LDH release, lipid peroxidation, glutathione levels, or ATP levels. Parameters were assessed as described previously in Chapter 1 Materials and Methods.

To investigate if pyruvate was acting as an energy substrate, experiments were performed with glucose treatment in place of pyruvate. Renal slices (50-100 mg) were co-incubated with glucose (10 mM or 1.67 mM final concentration) and myoglobin (final concentration 0, 4, 10 or 12 mg/mL) for two hours at 37^{o}C under 100% oxygen and constant shaking (100 cycles/minute). Because high concentrations of glucose can function as radical scavengers (Sagone *et al.*, 1983; Wehmeier and Mooradian, 1994), two concentrations were chosen. The lower concentration of the glucose applied, 1.67 mM, was the average amount that was measured in the control slices upon completion of a two-hour incubation. This amount was the maximum amount of glucose the slices

produced under control conditions. Upon completion of the incubations period, samples were analyzed for LDH release and gluconeogenesis.

Other experiments investigated the effect of the hydroxyl radical scavenger, dimethylthiourea (DMTU). Renal slices (50-100 mg) were co-incubated with DMTU (final concentration 0.1mM) and myoglobin (0, 4, 10 or 12 mg/mL final concentration) for 90 minutes at 37^{o}C under 100% oxygen and constant shaking (100 cycles/minute). At the end of the 90-minute incubation, gluconeogenesis was stimulated by addition of 100 μL of pyruvate (10 mM final concentration, in Krebs) and the tissue was incubated an additional 30 minutes. Upon completion of the incubations period, samples were analyzed for LDH release and gluconeogenesis.

2.3. RESULTS

As illustrated in Figure 14, pyruvate imparted protection against myoglobin toxicity. A concurrent incubation of 10 mM pyruvate with myoglobin for 120 minutes demonstrated protection against LDH release. When pyruvate was present for the last 30 minutes, a hint of protection was evident, albeit not significant. When pyruvate was present for the entire two-hour incubation, the protection was significant. Clearly, pyruvate attenuated LDH release.

2.3.1 Alternative substrate for energy

One possible mechanism for pyruvate mediated protection was that pyruvate served as an energy substrate. The effects of pyruvate on LDH release were compared to those of glucose. A two-hour co-incubation with glucose (10 mM) and myoglobin (0-12 mg/mL) resulted in a decrease of LDH release in the treated group as compared to the vehicle group (data not shown). However, a more relevant concentration of glucose (1.67 mM) failed to provide protection against myoglobin-induced LDH release (Figure 15). Furthermore, the concentration-dependent effect of myoglobin on LDH release was comparable between vehicle and glucose co-incubated tissues. Although the LDH release levels were significantly above control, there was a significant protection at the medium and high concentrations of myoglobin in the presence of pyruvate. In addition, pyruvate-treated tissue did not develop the myoglobin concentration-dependent rise in LDH release.

To address further the mechanism of pyruvate protection, ATP levels were measured in response to pyruvate treatment. Pyruvate induced increases in ATP levels

by five-fold even in the absence of myoglobin (Figure 16). However, slices concurrently treated with pyruvate and myoglobin, demonstrated incomplete protection of the decline of ATP levels of the myoglobin-treated group as compared to the control group. The ATP levels in the pyruvate-treated group incubated with 4 or 10 mg/mL myoglobin were not different from the pyruvate-treated control group. However, the pyruvate-treated group when incubated with 12 mg/mL demonstrated decreased ATP levels from the pyruvate-treated control group.

2.3.2 Detoxification of radical pathways

Another possible mechanism of pyruvate protection was that pyruvate was acting as a radical scavenger similar to dimethylthiourea (DMTU). The effect of myoglobin on LDH release and gluconeogenesis were investigated in the presence of DMTU. Slices concurrently treated with DMTU and myoglobin for two hours, continued to exhibit toxicity as measured by LDH release (Figure 17). A significant myoglobin concentration-dependent increase in LDH release was observed. Additionally, DMTU failed to protect against loss of gluconeogenesis (Figure 18). In the presence of DMTU, myoglobin still induced a loss of gluconeogenesis in a significant concentration-dependent manner. In fact, the levels of glucose were basically indistinguishable between DMTU and vehicle treated groups exposed to myoglobin.

The effects of pyruvate on myoglobin-induced lipid peroxidation and changes glutathione levels were investigated. Concurrent treatment of the slices with myoglobin and pyruvate revealed a significant protection against lipid peroxidation (Table 4). Pyruvate alone did not alter lipid peroxidation in the absence of myoglobin. In the

presence of pyruvate, lipid peroxidation was not increased in the myoglobin-treated group as compared to the control group. In addition, at myoglobin concentrations of 4, 10, and 12 mg/mL lipid peroxidation was significantly less in the presence of pyruvate than in the absence of pyruvate.

The effect of pyruvate on glutathione levels was more interesting. In the presence of pyruvate total glutathione levels in the myoglobin-treated group, continued to be significantly lower than control at one and two hours (Figure 19). However, comparison of the glutathione levels in the pyruvate and vehicle groups revealed a key finding: at one hour, the level of the pyruvate-treated control was significantly higher than that of the vehicle-treated control. At two hours, all the total glutathione levels of the pyruvate-treated group were greater than those levels of the vehicle-treated control. Pyruvate acted to increase the levels of total glutathione. Pyruvate also affected the levels of the glutathione disulfide (GSSG) in renal slices. At one hour, in the presence of pyruvate there was a decrease in the GSSG levels, albeit not a significant one (Figure 20). At two hours, though, pyruvate inhibited the myoglobin-induced elevation of GSSG levels. The levels of GSSG in the vehicle-treated group were twice that of the pyruvate-treated group at 12 mg/mL myoglobin. The pyruvate studies taken as a whole, demonstrated protection against myoglobin-induced LDH release, lipid peroxidation, and changes in glutathione levels. Although the levels of ATP in the pyruvate-treated groups were five times those in the vehicle treated groups, ATP levels continued to decline in the presence of myoglobin regardless of treatment.

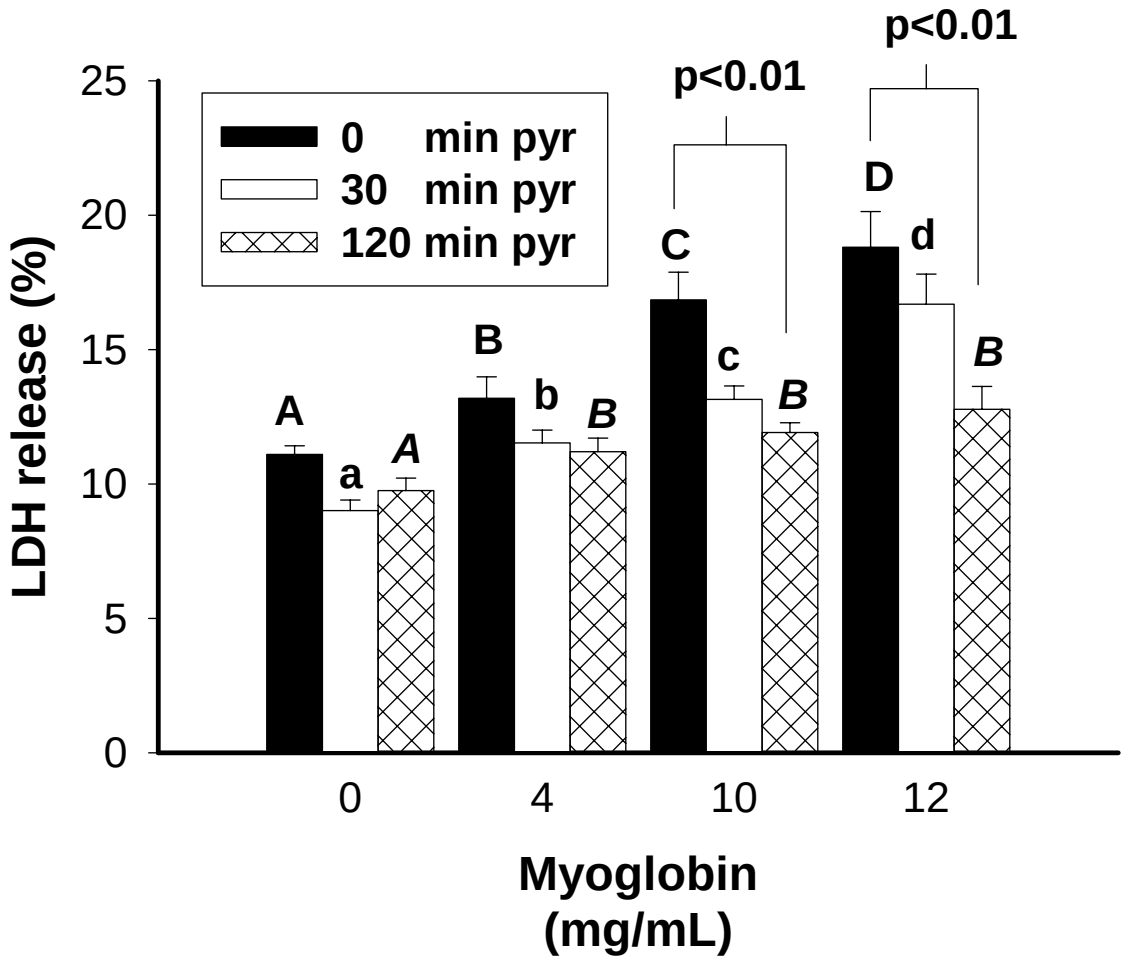


Figure 14. Effect of pyruvate on myoglobin-induced LDH release

This figure is a compilation of Figures 3 and 12. Renal cortical slices were incubated with myoglobin (0-12 mg/mL) for a total of 120 minutes at 37°C. Pyruvate (pyr, 10 mM final) was present for 0 minutes, the last 30 minutes, or the entire 120 minutes of incubation. LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

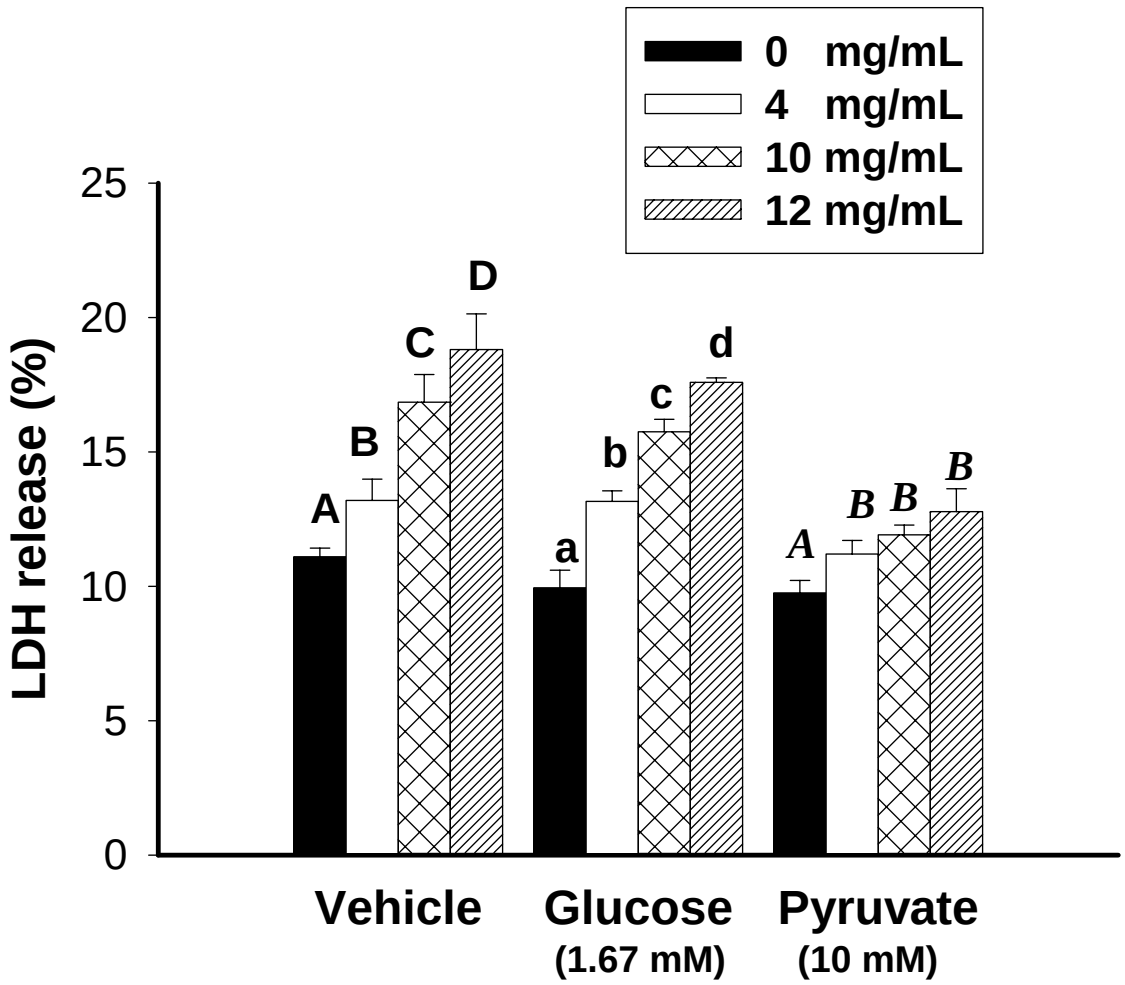


Figure 15. Effect of pyruvate or glucose on myoglobin-induced LDH release

Renal slices were incubated with pyruvate (10 mM final) or glucose (1.67mM final) and myoglobin (0-12 mg/mL) for 120 minutes at 37°C. LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

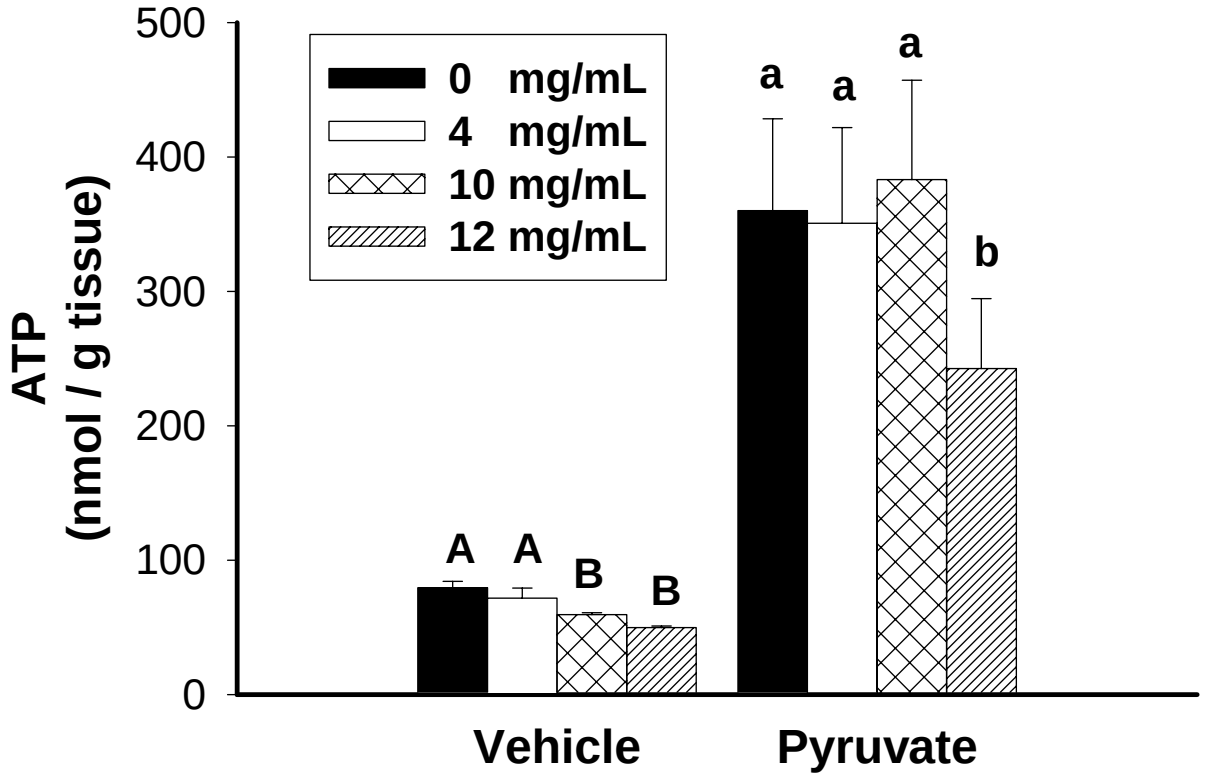


Figure 16. The effect of pyruvate on myoglobin-induced changes of ATP levels

Renal slices were co-incubated with pyruvate (10 mM final) and myoglobin (0-12 mg/mL) for 120 minutes at 37°C. Samples were extracted and analyzed for ATP. Data was expressed as amount ATP (nmol)/ g tissue. Values represent mean ± SEM with n=4-6 animals. Groups with dissimilar superscripts were statistically ($p<0.05$) different from one another within each treatment.

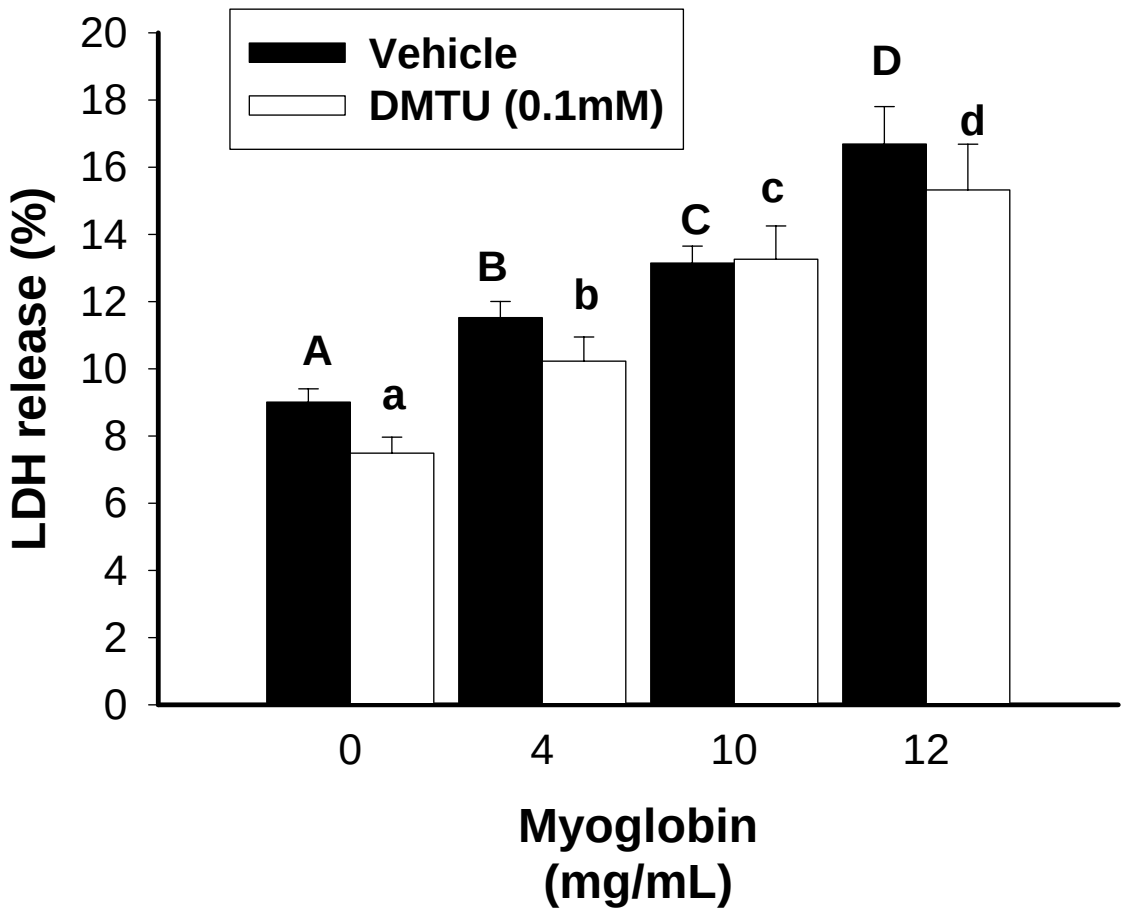


Figure 17. Effect of DMTU on myoglobin-induced LDH release

Renal slices were co-incubated with DMTU (0.1mM final) and myoglobin (0-12 mg/mL) for 120 minutes at 37°C. LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

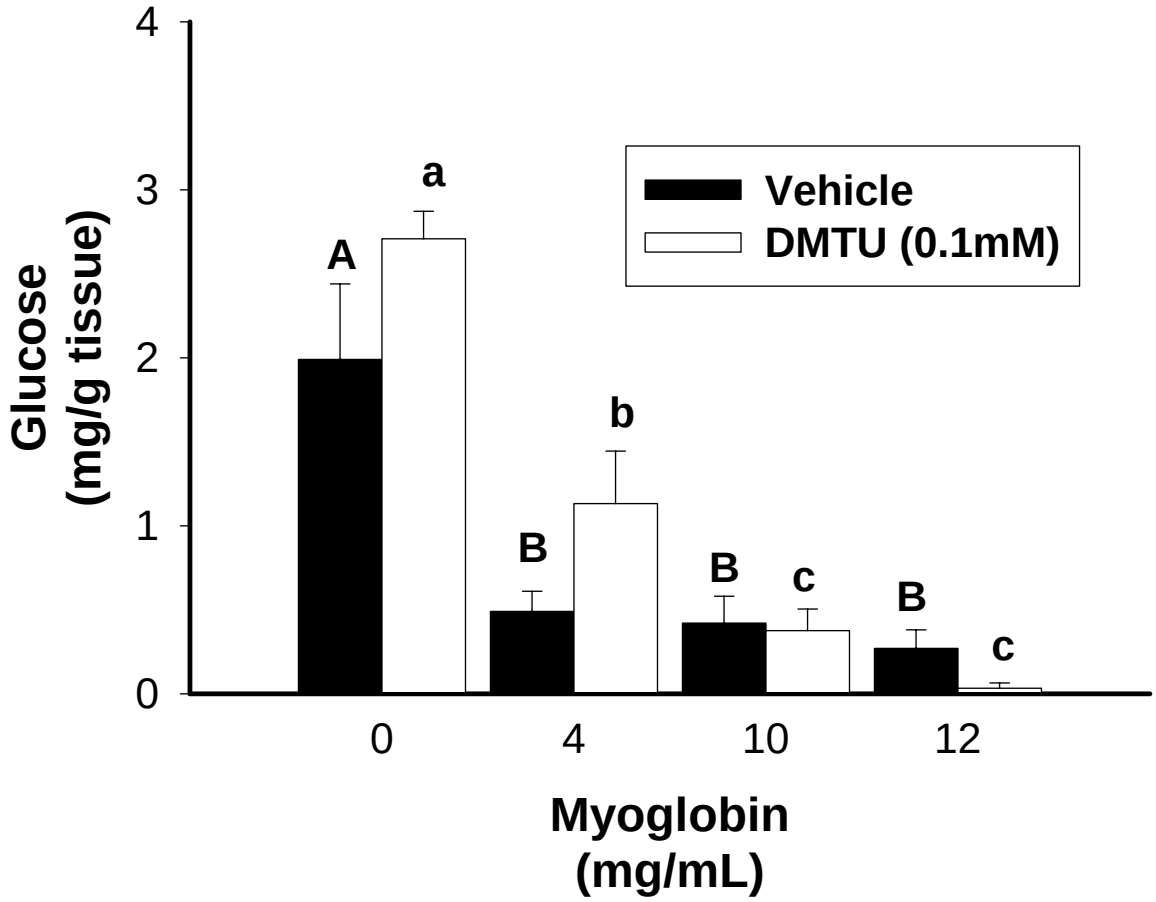


Figure 18. Effect of DMTU on myoglobin-induced loss of gluconeogenesis

Renal slices were co-incubated with DMTU (0.1mM final) and myoglobin (0-12 mg/mL) for 120 minutes at 37°C. To stimulate gluconeogenesis, pyruvate (10 mM final) was added during the last 30 minutes of incubation. Gluconeogenesis data are expressed as amount glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

Table 4. Effect of pyruvate on myoglobin-induced lipid peroxidation

Pyruvate (mM)	Myoglobin (mg/mL)			
	0	4	10	12
	nmol/g tissue	nmol/g tissue	nmol/g tissue	nmol/g tissue
0	114.20 ± 3.76^{A}	145.23 ± 9.09^{B}	135.60 ± 3.92^{B}	140.50 ± 6.34^{B}
10	95.63 ± 7.82^{A}	$108.92 \pm 5.05^{A*}$	$110.78 \pm 8.89^{A*}$	$111.58 \pm 7.53^{A*}$

Renal slices were co-incubated with pyruvate (0 or 10 mM final) and myoglobin (0-12 mg/mL) for 120 minutes at 37°C. Lipid peroxidation was measured as described in methods. Data are expressed as amount MDA (nmoles) per gram (g) tissue. Values represent mean ± SEM with n=4-6 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each incubation period. An asterisk (*) indicates statistical ($p<0.05$) difference between treatments with corresponding concentrations of myoglobin.

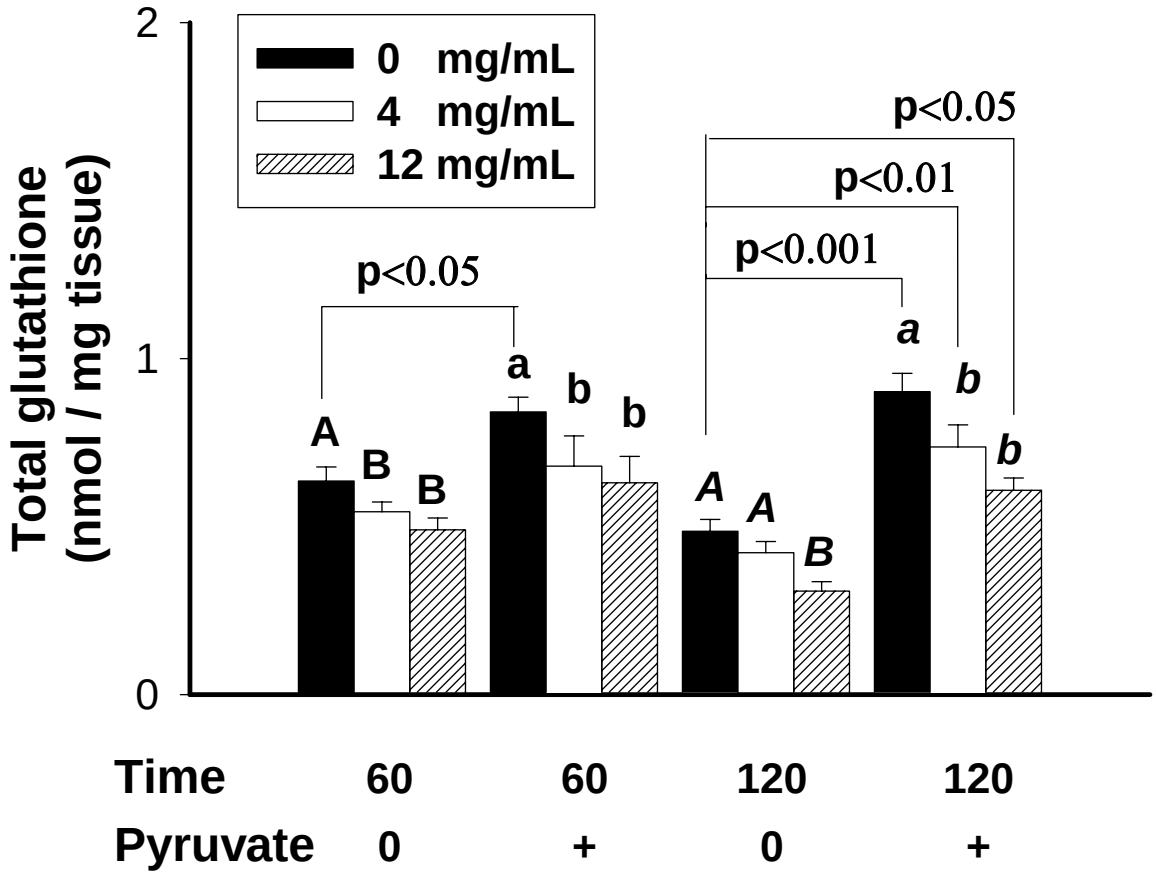


Figure 19. Effect of pyruvate on myoglobin-induced changes in total glutathione levels

Renal slices were co-incubated with pyruvate (0 or 10 mM final) and myoglobin (0-12 mg/mL) for 60 or 120 minutes at 37°C. Samples were extracted and analyzed for glutathione content as described in methods. Total glutathione data are expressed as nmol/ mg tissue. Values represent mean ± SEM with n=4-6 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each incubation period.

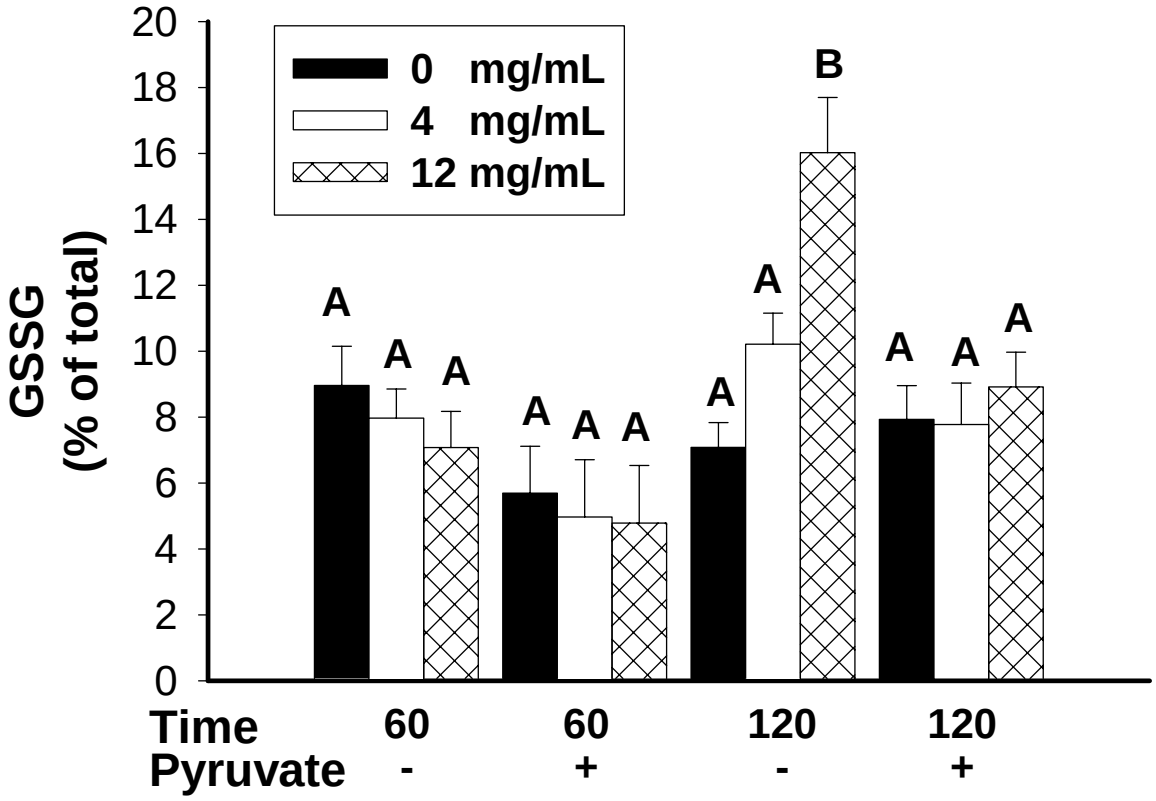


Figure 20. Effect of pyruvate on myoglobin-induced changes in GSSG levels

Renal slices were co-incubated with pyruvate (0 or 10 mM final) and myoglobin (0-12 mg/mL) for 60 or 120 minutes at 37°C. Samples were extracted and analyzed for GSSG content. Data are expressed as %GSSG of total glutathione. Values represent mean ± SEM with n=4-6 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each incubation time.

2.4. DISCUSSION

As illustrated in Figure 14, pyruvate protected against myoglobin toxicity. Pyruvate significantly reduced myoglobin toxicity when pyruvate was co-incubated with myoglobin and renal slices for two hours. Although there was no significant protection when pyruvate was present for only 30 minutes; clearly, pyruvate attenuated myoglobin-induced LDH release with respect to the amount of time that it was present in the incubation media. Two possibilities for the mechanism of pyruvate protection were originally proposed: pyruvate could be serving as an energy source or it could be detoxifying radical pathways.

2.4.1 Alternative substrate for energy

One possible mechanism of protection was that pyruvate acted as a substrate for energy production. This was addressed in protection assays with glucose and evaluation of ATP levels in the presence of pyruvate. For the protection assays with glucose, slices were concomitantly treated with myoglobin and glucose. A concentration of 10 mM glucose provided protection of myoglobin-induced LDH release (data not shown). Because at high concentrations, glucose can function as a radical scavenger (Sagone *et al.*, 1983; Wehmeier and Mooradian, 1994), a lower, more physiological concentration was chosen. The second concentration of the glucose applied, 1.67 mM, was the average content that was measured in the media of controls. This was the maximum amount of glucose the slices produced under control conditions. The effect of this concentration of glucose on myoglobin-induced LDH release was compared to those of pyruvate and vehicle. During a two-hour co-incubation with

glucose, myoglobin produced a concentration-dependent release of LDH (Figure 15). Furthermore, glucose failed to significantly reduce the LDH release of the myoglobin-treated group as compared to the vehicle-treated group, thus providing no protection against loss of membrane integrity. In addition, the myoglobin-induced concentration-dependent increase in LDH release of the glucose-treated group was comparable to the vehicle-treated group. With the pyruvate-treated tissue, there was significant protection at medium and high doses of myoglobin, whereas glucose did not afford protection. It is unlikely that the lack of protection at the low concentration of glucose was due to inaccessibility to glucose transporters in the lumen because glucose may be taken into the cells via a sodium-independent glucose transporter on the basolateral membrane (Silverman, 1986; Mullin *et al*., 1989). Although pyruvate may have slightly contributed to energy preservation, the data suggested another mechanism for protection.

To address further the mechanism of protection via an energy possibility, ATP levels were measured in response to pyruvate treatment (Figure 16). The overall five-fold increase of ATP levels in the pyruvate-treated group as compared to vehicle-treated group was an unexpected finding. Because pyruvate induced a five-fold increase in ATP levels, it is possible that this increase was one basis for the mechanism of pyruvate protection. However, slices concurrently treated with pyruvate and myoglobin, demonstrated incomplete protection against the decline of the ATP levels (Figure 16). At 4 and 10 mg/mL myoglobin, there was no difference in ATP levels of the pyruvate-treated group as compared to the pyruvate-treated control group. However, at 12mg/mL myoglobin, there was a significant decrease in the ATP levels as compared to control within the pyruvate-treated group. Pyruvate provided limited protection from

LDH release and the decline of ATP levels at the high concentration of myoglobin. The percentage of LDH release above control was the same percentage of decline below control in ATP levels when slices were incubated with pyruvate and myoglobin. This mimicked the experiments in the absence of pyruvate in that the percentage of LDH release above control in the myoglobin treated was the same percentage of decline below control in ATP levels. In consideration of the relationship between LDH release and ATP changes, the decline in ATP levels seems to have been caused by loss of membrane integrity. When taken with the glucose experiments, the changes in ATP levels indicated that pyruvate might have slightly acted to sustain energy, but also suggested an additional mechanism for protection.

2.4.2 Detoxification of radical pathways

As a second possibility, pyruvate could be acting to detoxify radical pathways. Because H_2O_2 levels are elevated in myoglobin toxicity and part of the mechanism is iron-dependent, hydroxyl radicals could be the major radical contributing to toxicity. Previous literature has reported variable results for the possible role of hydroxyl radical in myoglobin toxicity (Shah and Walker, 1988; Zager *et al.*, 1995; Zager and Burkhart, 1997). To ascertain the possibility that a hydroxyl radical is generated and to better explain the protection by pyruvate, assays were performed with a known hydroxyl radical scavenger, dimethylthiourea (DMTU). Slices were co-incubated with myoglobin and DMTU for two hours. DMTU did not afford any significant protection against LDH release and thus loss of viability (Figure 17). Furthermore, DMTU had no significant protective effect on gluconeogenesis. It is doubtful that the concentration of DMTU was

not sufficient to produce results, as the concentration used was within the range used in other *in vitro* studies (Zager and Burkhart, 1997). Overall, the lack of protection suggested that hydroxyl radicals were not involved in myoglobin toxicity in renal cortical slices within the selected time period. In addition, because pyruvate protected against LDH release and DMTU did not, these experiments suggested the mechanism of pyruvate protection did not involve detoxification of hydroxyl radicals. The previous DFX and GSH protection experiments together indicated that the loss of gluconeogenesis might be due to an iron-independent radical. This excluded the hydroxyl radical as a potential culprit. The lack of effect of DMTU to reduce myoglobin mediated effects on gluconeogenesis lent further support to a lack of hydroxyl radical involvement. This is to say that in the short-term exposure of myoglobin to renal cortical slices, hydroxyl radicals do not appear to have a role. However, the possibility remains that hydroxyl radicals may be involved at some point in rhabdomyolysis.

If pyruvate was acting to detoxify other radicals, the effect on lipid peroxidation and glutathione levels should reveal this. A two-hour incubation period was chosen although lipid peroxidation was first demonstrated at one hour. The rationale for this selection was: first, pyruvate may have increased the amount of time necessary for myoglobin to induce lipid peroxidation. Thus, an extra hour of incubation would allow more time to detect increases in lipid peroxidation. Second, at one hour, the variability of the data may have masked any protection. Co-incubation of the slices with myoglobin and pyruvate for two hours led to a significant decrease in lipid peroxidation as compared to control and vehicle-treated tissues (Table 4). This suggested that

pyruvate might be acting to detoxify radicals that are responsible for the lipid peroxidation in general.

The glutathione levels revealed a more interesting finding. A closer look at the glutathione data revealed an increase in total glutathione as compared to vehicle at all concentrations of myoglobin at both incubation times. Both a one and two-hour incubation with myoglobin and pyruvate continued to result in a decline of total glutathione levels as compared to control (Figure 19). This data suggested that perhaps, pyruvate was serving to increase the levels of total glutathione thus enabling the tissues to better manage oxidative damage. Pyruvate has been reported to enhance the endogenous glutathione system in myocardium (Tejero-Taldo *et al.,* 1999; Mallet, 2000). The increase in ATP levels in the presence of pyruvate could also contribute to the beneficial changes in total glutathione levels, by providing sufficient ATP to catalyze the conversion of oxidized glutathione to reduced glutathione. Concerning the glutathione disulfide (GSSG) levels, the overall increase of total glutathione may partially explain the reduction in the percentage GSSG with a two-hour incubation (Figure 20). Increasing the levels of total glutathione would decrease the percentage of GSSG as compared to total. In summary, pyruvate might have been working in conjunction with reduced glutathione to detoxify radicals and decrease lipid peroxidation. However, the decline in the total glutathione levels in the myoglobin treated versus control refuted this. Additionally, pyruvate appeared to increase glutathione levels and ATP levels, allowing greater capacity to deal with oxidative stress and perhaps to help fuel repair systems.

CHAPTER 3. THE ROLE OF IRON AND OTHER COMPONENTS OF MYOGLOBIN IN TOXICITY

3.1. INTRODUCTION

Iron is essential for many processes in the body; yet, it also plays a role in the formation of radicals that can attack biological molecules. Thus, control of iron levels is achieved via a tightly regulated system the body has developed. This control mechanism includes unique proteins for the transport (transferring) and storage (ferritin) of iron. The normal blood level of iron is 10μM (Ferreira *et al.*, 1999). In muscle cells, iron is maintained in the ferrous (Fe^{2+}) form and is unable to participate in redox cycling (Moore *et al.*, 1998). Under certain circumstances the iron in the heme group can redox cycle between the ferric (Fe^{3+}) and the ferryl ($[Fe=O]^{2+}$) forms. The latter of which may directly initiate lipid peroxidation (Hogg *et al.*, 1994; Patel *et al.*, 1996). The existence of the ferryl form has been questioned. However, recent evidence suggests that it does exist. Holt *et al.*, (1999) described evidence to indicate ferryl myoglobin exists *in vivo*. The heme present in the urine of rhabdomyolytic patients was cross-linked to protein. This process only occurs during the formation of the ferryl form. In order for the iron to redox cycle, it must first exist in the ferric (Fe^{3+}) form. Interestingly, it is estimated that at any given time *in vivo*, 1-2% of human hemoglobin is in the ferric form (Antonini and Brunori, 1971).

When the filtered load of myoglobin or hemoglobin exceeds absorptive capacity of the proximal tubules, the reabsorption of water results in an increase in the concentration of the globin. Together, this process results in the precipitation of proteins within the lumen and subsequent release of iron (Bunn and Jandl, 1969; Zager,

1992). The redox form deposited is metmyoglobin (Fe^{3+}) (Moore *et al.*, 1998). This metmyoglobin deposition could provide a mechanistic basis to explain how myoglobinuria can cause lipid peroxidation independent of free iron and conventional Fenton reactions (Moore *et al.*, 1998). Of course, this premise is based on the ability of the metmyoglobin to deposit in the lumen, thus initiating the toxicity. In the *in vitro* slice model, the lumen is collapsed. Any toxicity noted would preclude the necessity of deposition of metmyoglobin or the release of iron within the lumen for initiation of toxicity. Furthermore, the role of iron in lipid peroxidation *in vivo* is questionable because the body has developed comprehensive mechanisms to bind free iron and prevent toxicity.

An alternative first step in the free radical pathway is proximal tubule cell transport and accumulation of porphyrin molecules. In cells, the porphyrin rings can undergo degradation and result in iron release with the released iron being eliminated over several weeks (Bunn and Jandl, 1969). The iron can be liberated by various means including H_2O_2 (Gutteridge, 1986) and heme oxygenase. Heme oxygenase (HO) is a stress protein found predominately in the distal tubules. HO serves to break down heme and liberates free iron. It is increased in response to any stimulus producing oxidative stress. The HO-1 gene is upregulated exclusively in distal parts of the nephron while the proximal tubule sustains the damage. HO is rapidly upregulated in myoglobin-ARF and the induction or suppression can change the severity of glycerol-ARF (Nath *et al.*, 1992). Nath *et al.* (1992) reported that induction of HO with small concentrations of myoglobin protected renal function from subsequent rhabdomyolysis in the glycerol model using male Sprague Dawley rats. Likewise, Zager *et al.* (1995)

verified that incubation with an HO inhibitor could block the *in vitro* expression of increased MDA and LDH release levels following glycerol injection. Later, Zager and Burkhart (1997) further demonstrated that co-incubation of HK-2 cells with an HO inhibitor induced a concentration dependent cytoprotection suggesting that inhibition of the breakdown of myoglobin could enlist cytoprotection.

Tin protoporphyrin IX (SnPP) is a competitive inhibitor of HO. Studies have used SnPP to confer protection against myoglobin toxicity. Zager *et al.* (1995) reported that SnPP protected proximal tubule cells isolated from glycerol-injected animals from lipid peroxidation and cell death. He further reported that adding SnPP only during the *in vitro* part of the study still induced cytoprotection. Zager and Burkhart (1997) also investigated SnPP effects on myoglobin toxicity in HK-2 cells. He demonstrated a concentration-dependent cytoprotection when the cells were co-incubated with myoglobin and SnPP.

The proposed mechanism of protection of the SnPP is the inhibition of HO and thus prevention of the degradation of myoglobin and subsequent release of free iron. Many investigators suggest that free iron is the main cause of the myoglobin toxicity. However, the source of this free iron is debatable. Zager (1993) reported that following a myoglobin infusion of the kidney; 25 μg of heme per mg total protein in proximal tubular segments (PTS) was loaded into the proximal tubules. A bleomycin analysis revealed that 'catalytic' free iron was double the heme content. Zager concluded there was a secondary release of iron from intracellular stores.

Iron chelators, such as DFX, have been shown consistently to protect against myoglobin toxicity. In addition to the chelation of free iron, the protective effect has

been taken generally as evidence for participation of hydroxyl radicals. DFX has been shown to directly scavenge hydroxyl radicals in *in vitro* systems (Halliwell and Gutteridge, 1986). However, it is unlikely that DFX was acting in this capacity because DFX probably was not present in sufficient concentrations intracellularly to be intimately associated with the site of production of hydroxyl radicals, as hydroxyl radical scavengers must be, due to the extremely fast rate of reduction of the radical (Halliwell and Gutteridge, 1986). DFX has conferred protection in *in vivo* and *in vitro* models of myoglobin toxicity (Paller, 1988; Zager and Burkhart, 1997). Specifically, rats infused with myoglobin demonstrated a 24% drop in ATP levels that was blocked by the administration of DFX. It was concluded that myoglobin depleted adenylate pools via an iron-dependent mechanism (Zager, 1991). This conclusion may not be entirely accurate because DFX has another potential mechanism of protection. DFX is capable of reducing the ferryl form of myoglobin and its associated globin radical to the ferric form (Turner *et al.*, 1991). Regardless of the specific mechanism, DFX protection can provide insight into the mechanism of myoglobin toxicity. Either way, the use of DFX can indicate free radical mechanisms that involve an iron-dependent pathway.

As outlined in Chapter 1, DFX use in this study agreed with previous literature in that it conferred protection as seen by the attenuation of LDH release (Figure 6). The ability of DFX to reduce myoglobin toxicity was further assessed using ATP levels, GSH levels, gluconeogenesis, and lipid peroxidation. Moreover, to better dissect the role of iron in the toxicity, assays were conducted in the presence of equimolar amounts of ferric chloride as compared to myoglobin. The free iron solution was prepared in a manner similar to myoglobin. More specifically, an equimolar amount of ferric chloride

was prepared in the presence of 4 mM ascorbic acid, as used to reduce the myoglobin to the ferrous form. Previous literature as well as experiments performed in this study indicated that only the reduced form of myoglobin was toxic (Figure 2; Zager and Foerder, 1992; Zager and Burkhart, 1997). In a study by Zager and Foerder (1992), a mixture of Fe^{2+}/Fe^{3+} (4 mM total) was added to proximal tubular segments for 45 minutes. The addition of Fe^{2+}/Fe^{3+} produced marked cytotoxicity as defined by LDH release and lipid peroxidation. Fe^{2+} or Fe^{3+} alone each induced massive lipid peroxidation, but only Fe^{2+} caused LDH release. This study will compare the toxicity of the myoglobin to that of equimolar amounts of iron.

Myoglobin nephrotoxicity cannot be attributed solely to tissue iron loading (Zager, 1991). It has been shown that endocytotic protein uptake and not necessarily cell iron loading is largely responsible for nephrotoxicity of heme proteins (Zager *et al.*, 1987). Heme may be involved in toxicity since the heme content in the mitochondria was increased ten-fold following glycerol injection into rats (Nath *et al.*, 1998). Clearly, the potential for heme to contribute to the toxicity does exist. This study will investigate this potential further. Equimolar amounts of SnPP were used in place of myoglobin. The toxicity will be compared to that of the myoglobin in its entirety and to the iron. Evaluating the toxicity of myoglobin as a whole and its components will give insight into the mechanism of myoglobin toxicity. In addition, protection studies with SnPP and DFX will strengthen the findings regarding the role of myoglobin and its components.

3.2. MATERIALS AND METHODS

3.2.1 Chemicals and sources

(Company and catalog number)

Sn(IV) Protoporphyrin IX (Frontier Scientific; Porphyrin Products, Sn749-9)
$FeCl_3$ (Fisher Scientific, I-88)
Protoporphyrin IX (Sigma, P8293)

3.2.2 Incubation of renal slices.

Renal cortical slices (50-100 mg) were pretreated for 10 or 15 minutes with DFX (0.1mM final concentration). Note: the pretreatment was 10 minutes for the LDH release and gluconeogenesis data, while 15 minutes with the other parameters of toxicity. Upon completion of the pretreatment period, myoglobin was added at a final bath concentration of 0, 4, 10 or 12 mg/mL. Tissues were incubated for two hours at 37^{o}C under 100% oxygen and constant shaking (100 cycles/minute) in a Dubnoff metabolic incubator. For the gluconeogenesis experiments, pyruvate (10 mM final concentration) was present for the last 30 minutes of incubation. Upon completion of the incubation, LDH release, pyruvate-stimulated gluconeogenesis, ATP levels, glutathione status, or lipid peroxidation were measured in vehicle and myoglobin-treated tissue. Parameters were assessed as described previously in Chapter 1 Materials and Methods.

Other experiments were designed as a component analysis of myoglobin. Renal slices (50-100 mg) were incubated with ferric chloride, protoporphyrin IX (PP9), or tin protoporphyrin (SnPP) at equimolar concentrations to myoglobin (0-12 mg/mL) for 90 minutes at 37^{o}C under oxygen and constant shaking (100 cycles/minute). At the end of

the 90-minute incubation, gluconeogenesis was stimulated by addition of 100 μL of pyruvate (10 mM final concentration, in Krebs) and the tissue was incubated an additional 30 minutes. See Appendix I for details on preparation of ferric chloride, PP9, and SnPP solutions in addition to detailed incubation procedures.

Additional studies investigated the pretreatment of renal slices with SnPP (0.03 mM final concentration) for 30 minutes prior to addition of myoglobin. Following pretreatment, myoglobin (0, 4, 10 or 12 mg/mL final concentration) was added and the slices were incubated for 90 minutes at 37^{o}C under oxygen and constant shaking (100 cycles/minute). At the end of the 90-minute incubation, gluconeogenesis was stimulated by addition of 100 μL of pyruvate (10 mM final concentration, in Krebs) and the tissue was incubated an additional 30 minutes.

3.3. RESULTS

Pretreatment with deferoxamine (DFX) did lend protection against myoglobin toxicity. Pretreatment of the slices for 15 minutes with DFX, followed by a two-hour incubation with myoglobin resulted in a significant attenuation of LDH release (Figure 6). The levels in the presence of myoglobin were not significantly different from control within the DFX treatment. Moreover, at 12 mg/mL myoglobin, a significant reduction in LDH release was evident as compared to vehicle treated. The gluconeogenesis data revealed an opposite finding. Pretreatment of the slices with DFX failed to protect against the loss of gluconeogenesis (Figure 7). The concentration-dependent decrease in gluconeogenesis induced by myoglobin was basically indistinguishable between the DFX and vehicle pretreated groups. Because DFX has varying effects of myoglobin toxicity, the role of DFX in the mechanism of myoglobin toxicity was further investigated by evaluating the effect on ATP levels, lipid peroxidation, and glutathione levels.

3.3.1 The effect of DFX on the parameters of myoglobin toxicity

Pretreatment of the slices with DFX for 10 minutes prior to a two-hour incubation with myoglobin prevented a decline of ATP levels (Figure 21). ATP levels were decreased at 10 and 12 mg/mL myoglobin in the absence of DFX. DFX pretreatment had a similar effect on myoglobin-induced effects on lipid peroxidation. In the presence of DFX, lipid peroxidation was markedly and significantly decreased as compared to control (Table 5). Furthermore, protection was afforded by DFX at all concentrations of myoglobin compared to vehicle-treated groups. At the high concentration of myoglobin, the levels of MDA were approximately 140nmoles per gram tissue whereas in the DFX

treated the levels were only about 100nmoles per gram tissue. Even the control values were significantly reduced: approximately 114nmoles per gram tissue in vehicle treated whereas about 84nmoles per gram tissue in the DFX pretreated group. Like lipid peroxidation, glutathione levels were dramatically protected. Myoglobin failed to decrease total glutathione levels below control in the presence of DFX (Figure 22). Additionally, the DFX pretreatment lead to a significant increase in levels of total glutathione at each concentration with respect to vehicle treated. Notably, this was similar to pyruvate protection as it increased total glutathione at all concentrations of myoglobin (Figure 19). The exception was a significant concentration-dependent decrease in total glutathione levels below control with the pyruvate treatment.

DFX also afforded protection of the rise in GSSG levels (Figure 22). In the presence of DFX, GSSG levels of the myoglobin treated were not significantly different from control. At 4, 10, and 12 mg/mL myoglobin, DFX pretreatment produced a significant decrease in the GSSG levels as compared to the DFX-vehicle group. In fact, at the high concentration of myoglobin, DFX pretreatment resulted in a striking four-fold decrease in the GSSG levels from the DFX-vehicle-pretreated group. Again, this result was similar to pyruvate protection, except the decrease was only two-fold (Figure 20). Also, there was a non-significant trend for all the GSSG levels of the DFX treated to be lower than the DFX-vehicle control group values.

3.3.2 Component analysis

As a part of the component analysis of myoglobin, ferric chloride ($FeCl_3$) was used to simulate the free iron contribution. Slices were incubated two hours with

equimolar amounts of $FeCl_3$ pretreated with ascorbic acid as compared to myoglobin. Incubation with $FeCl_3$ produced a significant concentration-dependent increase in LDH release (Figure 23.) The levels within each concentration of $FeCl_3$ were similar to those demonstrated with equimolar concentrations of myoglobin, albeit slightly lower. A difference between the treatments at each concentration was not evident until the highest concentration of myoglobin. At the high concentration, myoglobin produced a significantly elevated LDH release. However, remarkable differences in toxicity profiles between iron and myoglobin was revealed in the gluconeogenesis data (Figure 24). $FeCl_3$ produced a significant concentration-dependent compromise of gluconeogenesis; however, not as pronounced as observed for myoglobin. Additionally, at all concentrations of treatment (with the exception of the control group) there was a significant decrease in gluconeogenesis with the myoglobin treated as compared to the $FeCl_3$ treated groups. Although $FeCl_3$ resulted in a slight compromise, gluconeogenesis predominately was maintained which was in great contrast to the effect of myoglobin on gluconeogenesis. These results supported an iron-independent component to myoglobin toxicity.

Another part of the component analysis involved tin (Sn) protoporphyrin (IX) (SnPP) in place of myoglobin to simulate the heme component in the absence of iron. Protoporphyrin IX was originally chosen to simulate the heme component of myoglobin. However, due to solubility problems at levels comparable to 1mg/mL myoglobin, the studies were conducted with SnPP instead. Incubation of the renal slices with SnPP produced effects varying from those with myoglobin. The concentrations of SnPP used ranged from 0 to 0.227mM. These concentrations were equimolar to 0, 1, 2, 4mg/mL

concentrations of myoglobin. Incubation of the renal slices with SnPP produced a significant concentration-dependent decrease in LDH release in the treated groups as compared to control (Figure 27). This effect was in direct contrast to the myoglobin-induced increase in LDH release. To determine if SnPP was interfering with the LDH assay, the total levels of LDH were calculated. SnPP failed to have any effect on the total LDH values (Table 6).

The effects of SnPP on gluconeogenesis were similar to those effects of myoglobin. SnPP incubation of the renal slices produced a significant concentration-dependent decrease in gluconeogenesis, as did myoglobin treated (Figure 28). Note: the levels of myoglobin shown in the figure were 0-12 mg/mL because there were no gluconeogenesis data for 1 or 2 mg/mL myoglobin. The controls for the SnPP-treated group were lower than the myoglobin treated group, although not significant. Notably, the vehicle for the SnPP control was DMSO whereas the myoglobin vehicle was Krebs-Ringer buffer.

The third and final element of the component analysis was the utilization of SnPP in protection assays. SnPP inhibits heme oxygenase, the enzyme responsible for the breakdown of myoglobin as well as other globins. A thirty-minute pretreatment of slices with SnPP followed by a two-hour incubation with myoglobin, produced a significant concentration-dependent release of LDH as compared to control group values (Figure 25). However, the amount of LDH release was slightly lower in the SnPP treated as compared to the vehicle treated group. Specifically, at four and 10 mg/mL of myoglobin, there was a significant difference between the treatments. The protection lent by SnPP was more pronounced in the gluconeogenesis data (Figure 26). Within the SnPP

treated, the levels of gluconeogenesis in the presence of myoglobin were not significantly different from control. Furthermore, pretreatment with SnPP produced a significant rescue of the loss in gluconeogenesis at all levels of myoglobin as compared to SnPP-vehicle pretreatment. In summary, SnPP produced a slight protection of LDH leakage with a dramatic protection of gluconeogenesis.

In summary, DFX protected against the early events including lipid peroxidation and changes in glutathione levels in addition to the late events of LDH release and alterations of ATP levels. Ferric chloride produced a similar increase in LDH release as myoglobin, albeit, the levels were slightly lower. However, incubation of renal slices with SnPP yielded a protection of gluconeogenesis as compared to myoglobin treated. Pretreatment of the renal slices with a small concentration of SnPP prior to incubation with myoglobin demonstrated only a slight protection of LDH release with a considerable protection of gluconeogenesis. Incubation of the renal slices with higher concentrations of SnPP in the absence of myoglobin resulted in a significant decrease in LDH release and a similar loss of gluconeogenesis as compared to myoglobin treated tissue.

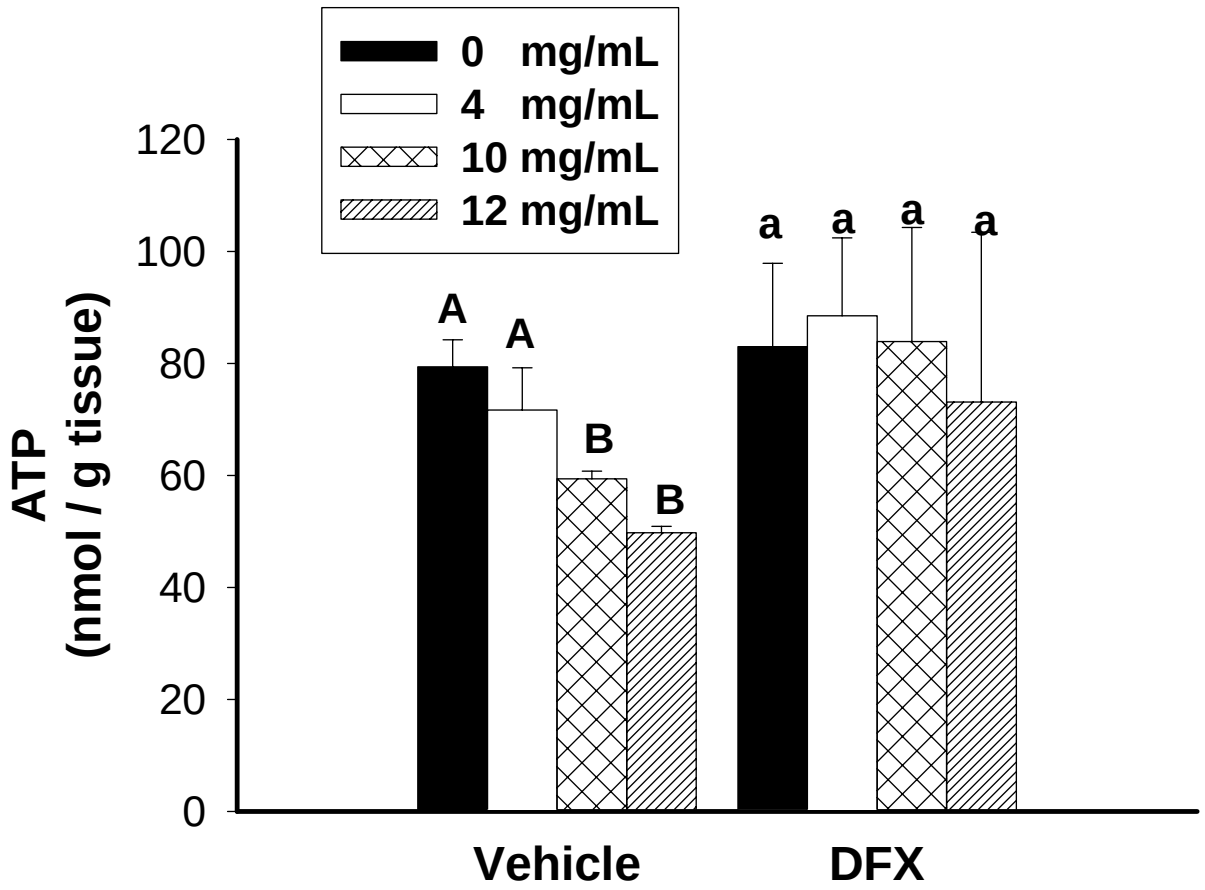


Figure 21. Effect of DFX on myoglobin-induced changes in ATP levels

Renal slices were pretreated with 0.1mM DFX for 10 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). Samples were extracted and analyzed for ATP. Data are expressed as ATP (nmol) / g tissue. Values represent mean ± SEM with n=4-6 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

Table 5. Effect of DFX on myoglobin-induced lipid peroxidation

DFX (mM)	Myoglobin (mg/mL) 0	4	10	12
	nmol/g tissue	nmol/g tissue	nmol/g tissue	nmol/g tissue
0	114.20 ± 3.76^{A}	145.23 ± 9.09^{B}	135.60 ± 3.92^{B}	140.50 ± 6.34^{B}
0.1	$84.29 \pm 3.23^{A*}$	$91.26 \pm 5.36^{A*}$	$92.36 \pm 7.45^{A*}$	$99.96 \pm 5.75^{A*}$

Renal slices were pretreated with 0.1mM DFX for 10 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). Data are expressed as nmoles MDA per gram (g) tissue. Values represent mean ± SEM with n=4-6 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment. An asterisk (*) indicates statistical ($p<0.05$) difference between treatments within corresponding concentrations of myoglobin.

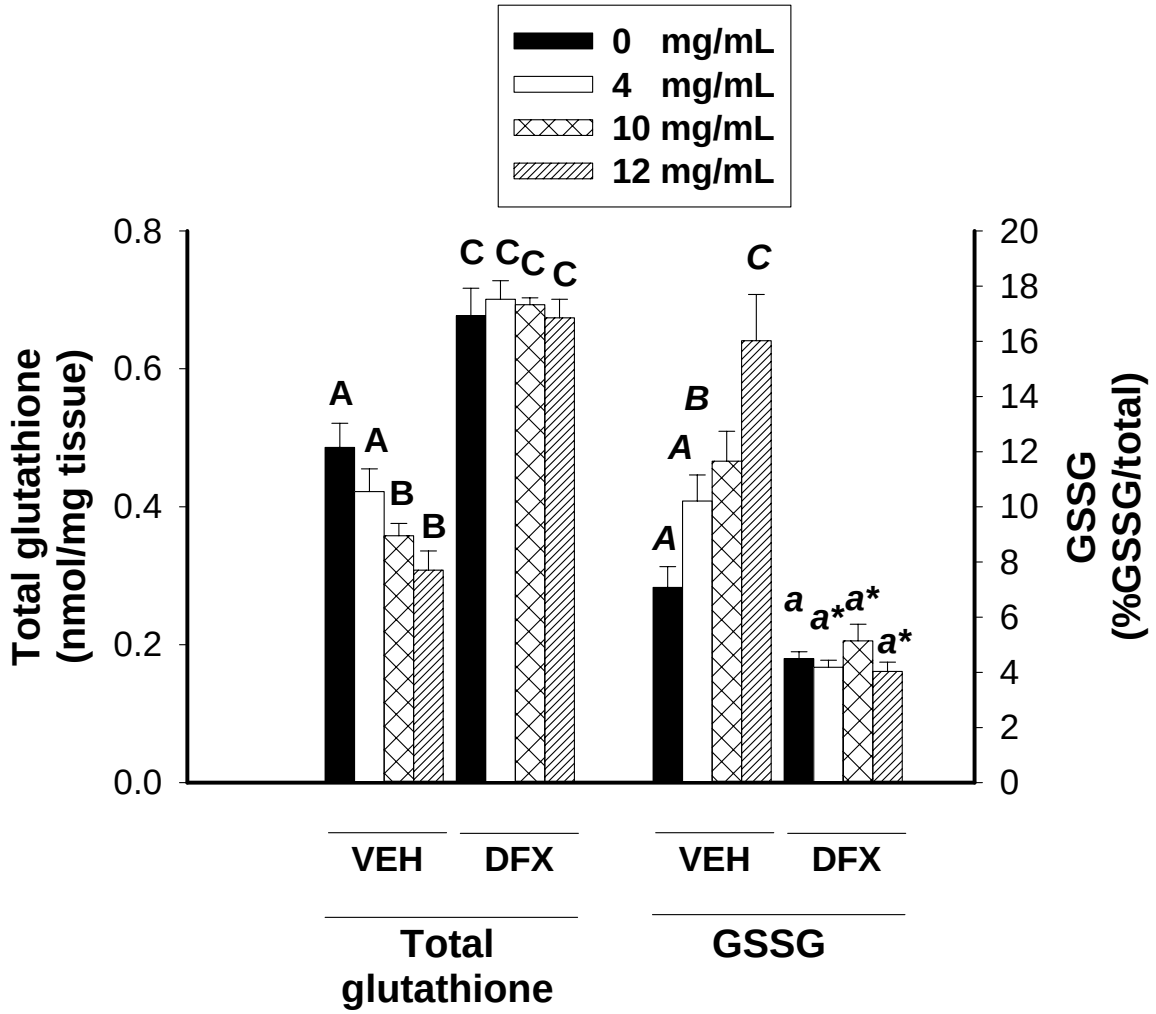


Figure 22. Effect of DFX on myoglobin-induced changes in glutathione levels

Renal slices were pretreated with 0.1mM DFX for 10 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). Samples were extracted and analyzed for glutathione content as described in the methods. Total glutathione data are expressed as nmoles of total per (mg) tissue. Within total glutathione levels, groups with dissimilar superscripts are statistically ($p<0.05$) different from one another. Glutathione disulfide (GSSG) data are expressed as %GSSG of total GSSG. Values represent mean ± SEM with n=4 animals. Within GSSG levels, groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each pretreatment. An asterisk (*) indicates statistical ($p<0.05$) difference of GSSG levels between treatments within corresponding concentrations of myoglobin.

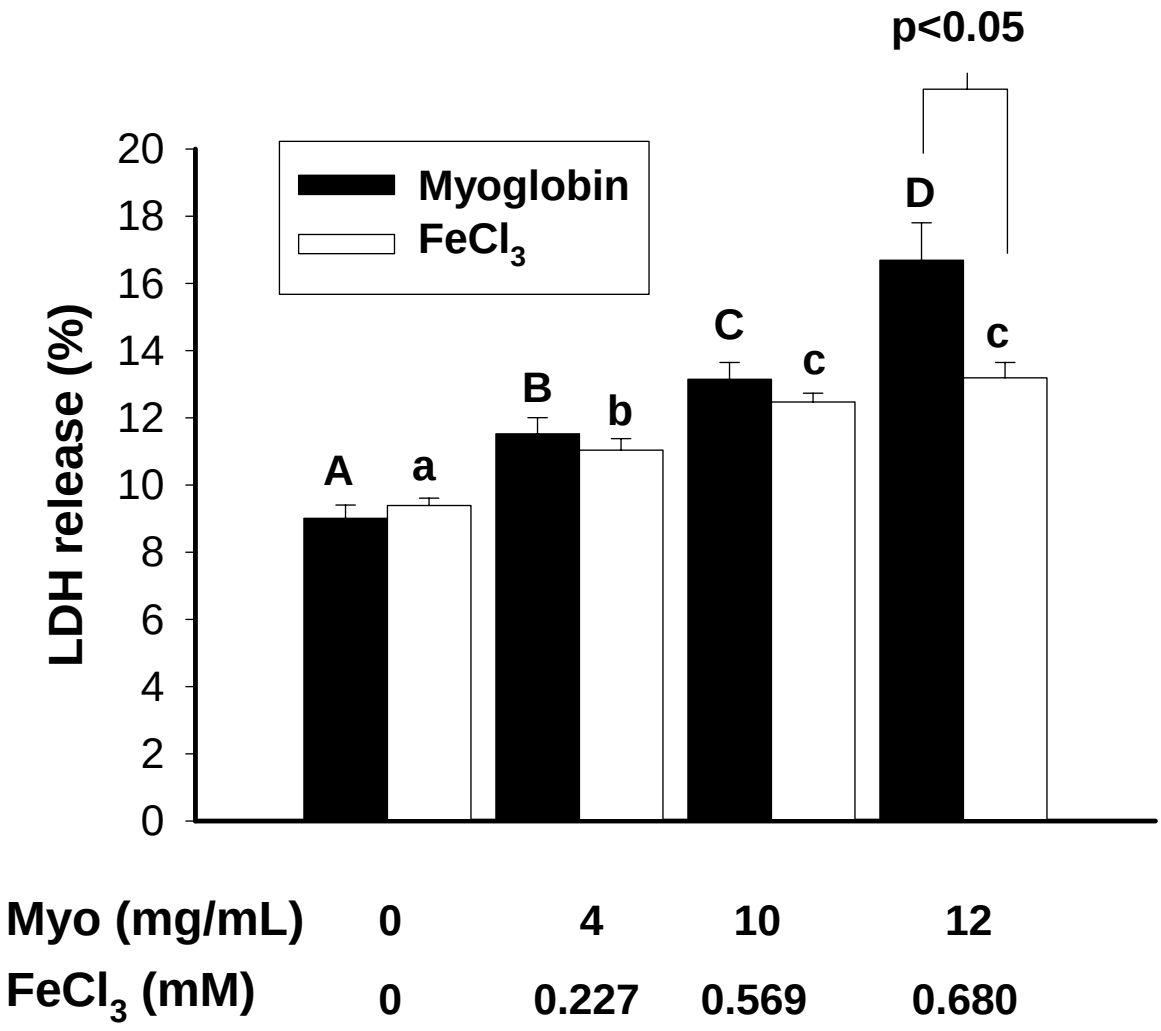


Figure 23. Comparison of LDH release in response to myoglobin or $FeCl_3$ incubation

Renal slices were incubated 120 minutes with myoglobin (0-12 mg/mL) or $FeCl_3$ (0-0.680 mM pretreated with 4 mM ascorbic acid). LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

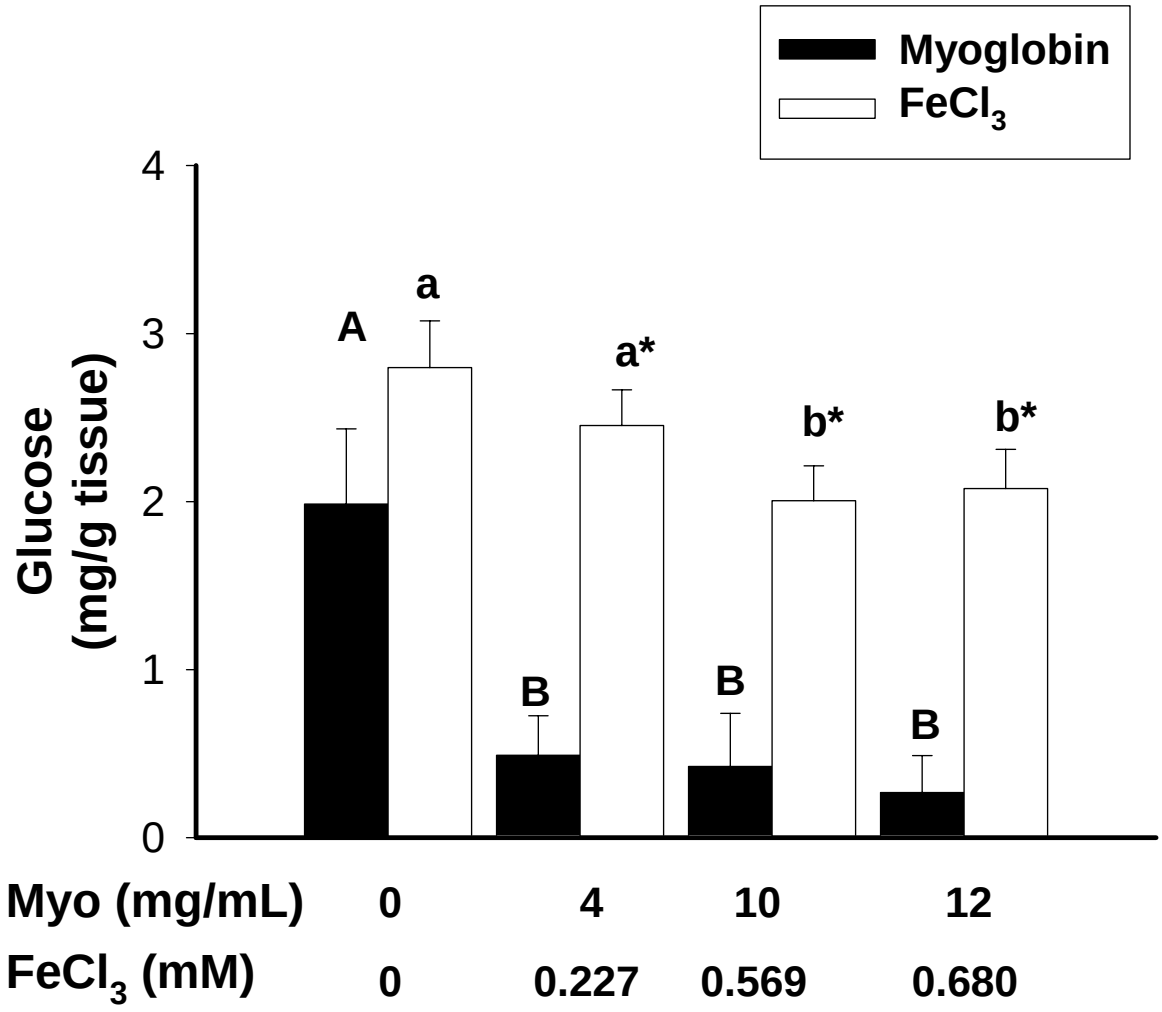


Figure 24. Comparison of gluconeogenesis in response to myoglobin or $FeCl_3$ incubation

Renal slices were incubated for 120 minutes with myoglobin (0-12 mg/mL) or $FeCl_3$ (0-0.680 mM pretreated with 4 mM ascorbic acid). To stimulate gluconeogenesis, pyruvate (10 mM final) was added during the last 30 minutes of incubation. Gluconeogenesis data are expressed as amount glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment. An asterisk (*) indicates statistical ($p<0.001$) difference between treatments within corresponding concentrations.

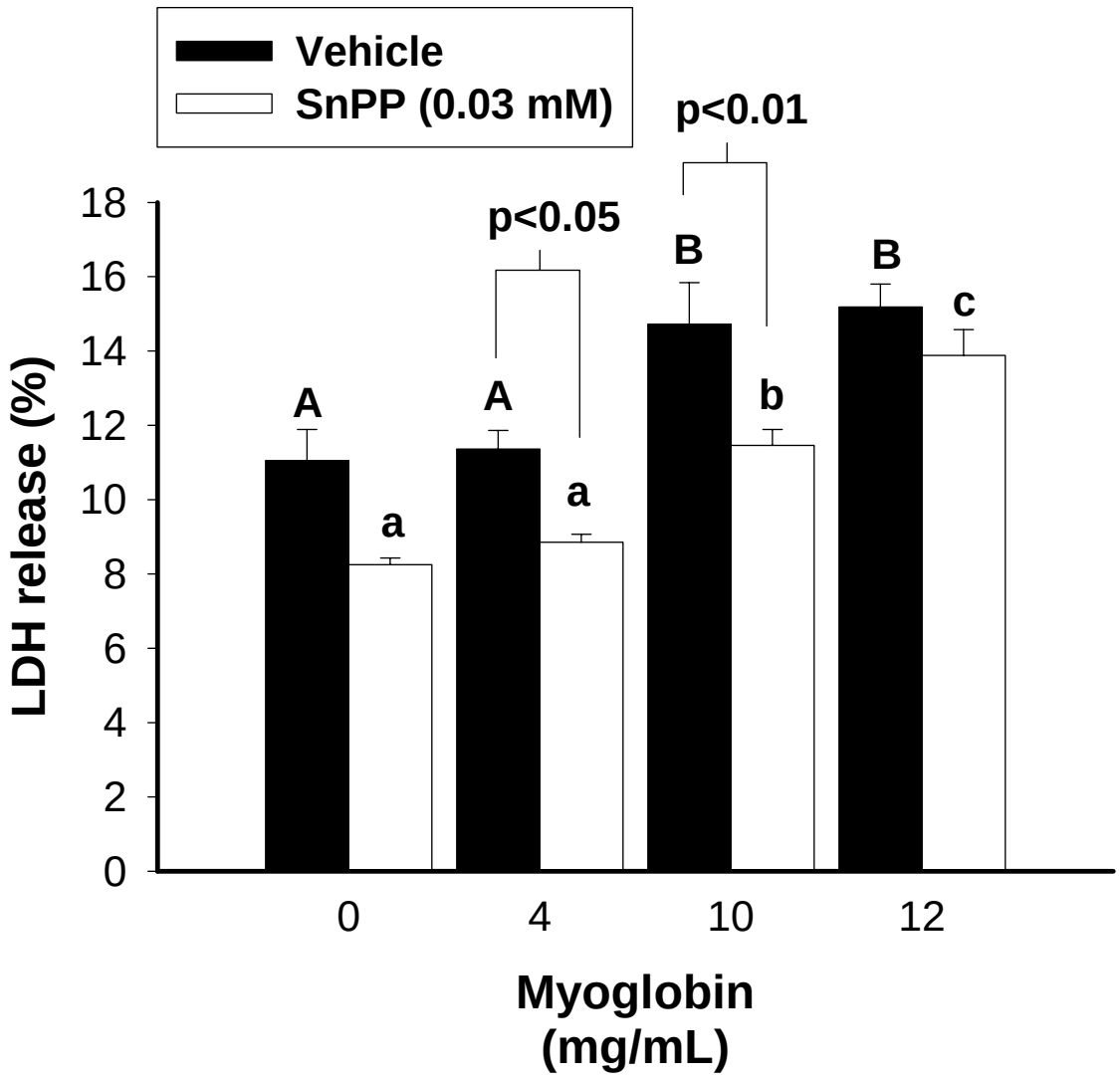


Figure 25. Effect of SnPP on myoglobin-induced LDH release

Renal slices were pretreated with 0.03 mM SnPP for 30 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each pretreatment.

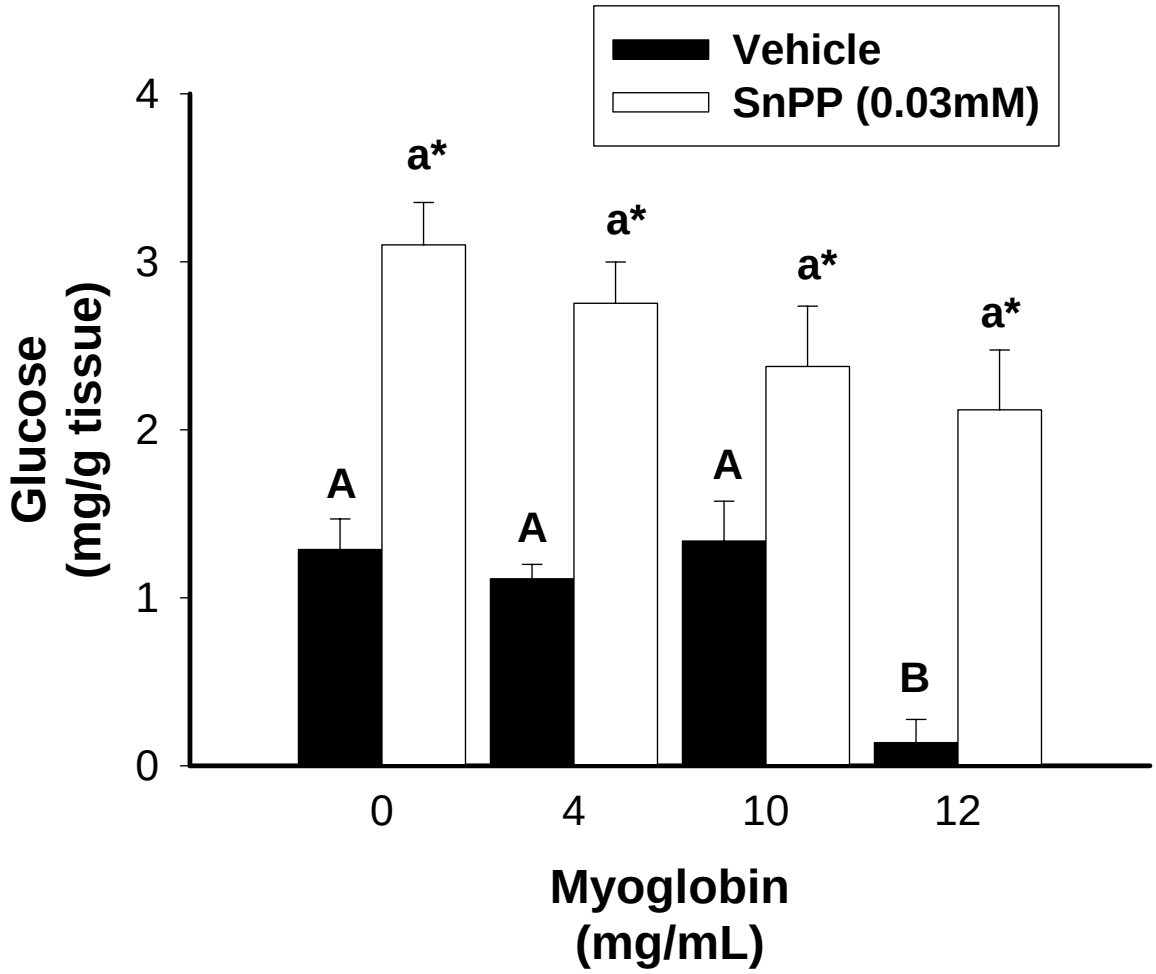


Figure 26. Effect of SnPP on myoglobin-induced loss of gluconeogenesis

Renal slices were pretreated with 0.03 mM SnPP for 30 minutes at 37°C. Following pretreatment, the tissues were co-incubated an additional 120 minutes with myoglobin (0-12 mg/mL). To stimulate gluconeogenesis, pyruvate (10 mM final) was added during the last 30 minutes of incubation. Gluconeogenesis data are expressed as amount glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment. An asterisk (*) indicates statistical ($p<0.05$) difference between pretreatments within corresponding concentrations of myoglobin.

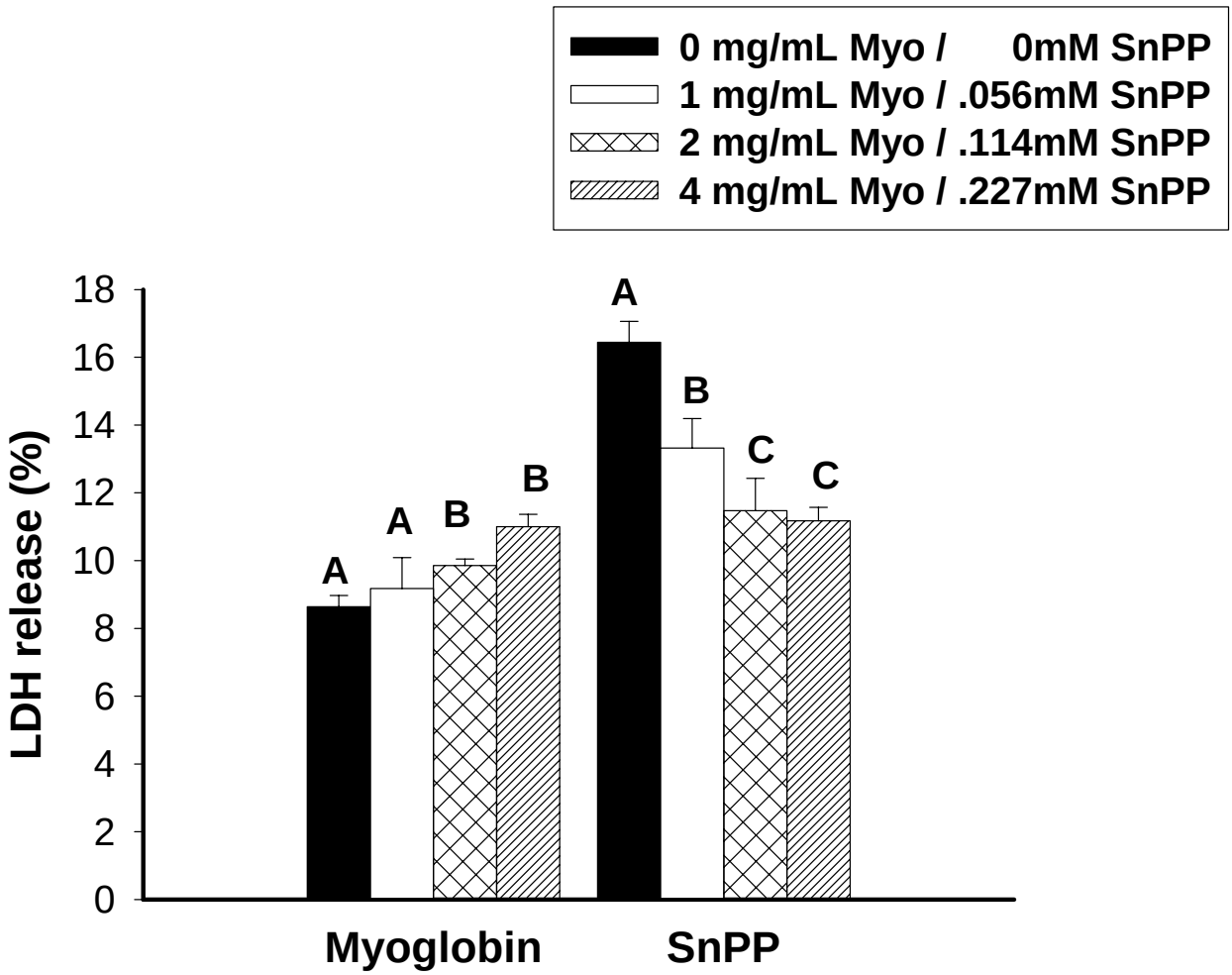


Figure 27. LDH release in response to incubation with myoglobin or SnPP

Renal slices were incubated 120 minutes with myoglobin (0-12 mg/mL, buffer vehicle) or SnPP (0-0.227 mM, DMSO vehicle). LDH release data are expressed as percent of total. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

Table 6. Total LDH following incubation of renal slices with SnPP

SnPP (mM)	Total LDH (Units) / mg tissue
0	25.7 ± 1.6^{A}
0.056	25.5 ± 1.7^{A}
0.114	27.0 ± 1.7^{A}
0.227	27.3 ± 0.8^{A}

Renal slices were incubated for 120 minutes with SnPP (0-0.227 mM). Total LDH was measured. Data are expressed as total LDH (units) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts are statistically ($p<0.05$) different from one another within each treatment.

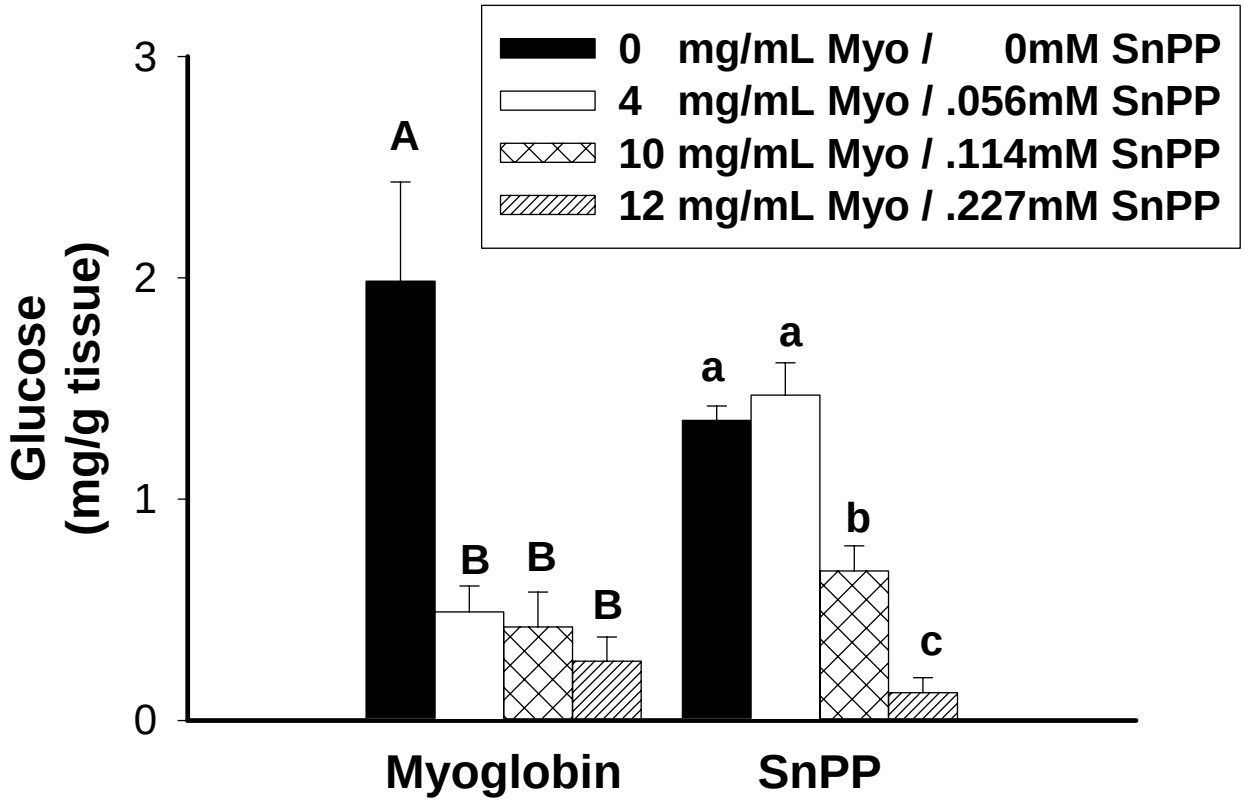


Figure 28. Gluconeogenesis in response to incubation with myoglobin or SnPP

Renal slices were incubated 120 minutes with myoglobin (0-12 mg/mL, vehicle water) or SnPP (0-0.227mM, vehicle DMSO). Note: SnPP levels are equimolar to 0,1,2 or 4 mg/mL myoglobin. To stimulate gluconeogenesis, pyruvate (10 mM final) was added during the last 30 minutes of incubation. Gluconeogenesis data was expressed as amount glucose (mg) per gram (g) tissue. Values represent mean ± SEM with n=4 animals. Groups with dissimilar superscripts were statistically ($p<0.05$) different from one another within each treatment.

3.4. DISCUSSION

Some form of iron has been shown repeatedly to be an important component of myoglobin toxicity. This chapter was designed to investigate this role as well as the role of the other components of myoglobin.

3.4.1 The effect of DFX on the parameters of myoglobin toxicity

Iron chelators, such as DFX, have been shown to protect against myoglobin toxicity in *in vivo* and *in vitro* models (Paller, 1988; Zager and Burkhart, 1997). The present study sought to investigate the protection with DFX to verify the slice model as a relevant model for studying myoglobin toxicity. Furthermore, the use of DFX would allow investigation of individual toxic events along the constructed time line in relation to iron involvement. DFX was an ideal iron chelator to use for this study for two reasons. Not only can DFX chelate free ferrous iron, but DXF is also capable of reducing the ferryl form of myoglobin and the associated globin radical to the ferric form (Turner *et al.*, 1991). In addition, DFX can directly scavenge hydroxyl radicals in *in vitro* systems (Halliwell and Gutteridge, 1986) or it can chelate iron used in the production of hydroxyl radicals. Regardless of the specific mechanism, DFX protection can provide insight into the iron-related mechanisms of myoglobin toxicity and the involvement of radicals.

DFX attenuated myoglobin toxicity (Figure 6). It was unlikely that DXF was directly scavenging hydroxyl radicals because it probably was not present in sufficient concentrations intracellularly to be intimately associated with the site of production of hydroxyl radicals. As a second possibility, DFX could have been chelating free iron, thus preventing the production of hydroxyl radicals propagated via Fenton reactions.

However, taken with the DMTU protection results, it is unlikely that DFX was functioning to decrease hydroxyl radicals in this way because the results with DMTU suggested hydroxyl radicals were not involved in myoglobin toxicity in the renal cortical slice model.

On the contrary, DFX pretreatment did not provide protection from myoglobin-induced decreases in gluconeogenesis (Figure 7). In fact, the fall of the glucose levels was basically indistinguishable between DFX and DFX-vehicle pretreated groups. The effects of DFX suggested several things concerning gluconeogenesis and myoglobin toxicity. For one, the loss of gluconeogenesis was not iron-dependent. Moreover, because DFX can detoxify ferryl myoglobin radicals, the results suggested that the effect on gluconeogenesis was not ferryl radical related. Also, because there was a dramatic loss of gluconeogenesis with retention of membrane integrity, loss of gluconeogenesis probably was not a major contributing factor to loss of viability. In general, the effect of DFX on LDH release indicated that the mechanism of toxicity was iron-dependent and based on the observed effects on gluconeogenesis, a component of the myoglobin toxicity was iron-independent.

Because there was lack of protection of one of the early indicators of toxicity, namely gluconeogenesis, the effect of DFX on the other early measures of toxicity was investigated. Together, lipid peroxidation and decreased glutathione levels implied the involvement of radicals. If these radicals were iron dependent, DFX should lend protection. Slices incubated with myoglobin following a 10-minute pretreatment with DFX were evaluated for lipid peroxidation. DFX significantly protected at all concentrations of myoglobin in addition to the controls (Table 5). The effect of DFX on glutathione levels mimicked that of lipid peroxidation. In the presence of DFX, slices

demonstrated an overall increase in total glutathione levels with a reduction in GSSG levels as compared to vehicle-treated group values (Figure 22). This was similar to the effect of pyruvate (Figure 19). However, whereas pyruvate did not protect against a decline of total glutathione levels within the treated groups, DFX provided significant protection. Like pyruvate, DFX prevented the increase in GSSG levels (Figure 22). At the high concentration of myoglobin, pyruvate (Figure 20) provided a two-fold reduction in the GSSG levels as compared to control; whereas, DFX afforded a four-fold reduction. Notably, pyruvate and DFX acted similarly, but not synonymously. Together, these results suggested that unlike pyruvate, DFX might have been acting to detoxify free radicals. This detoxification could have resulted in elevated total glutathione levels, as there were no more free radicals to detoxify.

The protection from lipid peroxidation and depletion of glutathione levels in the presence of DFX suggested these events were iron-dependent. In addition, the results implied that the radicals involved could have been the ferryl myoglobin radicals and/or other iron-dependent radicals barring hydroxyl radicals.

A study by Zager (1991) reported that myoglobin depleted adenylate pools via an iron-dependent mechanism. To determine if iron played a role in the decline of ATP levels, slices pretreated with DFX prior to a two-hour incubation with myoglobin were evaluated for changes in ATP levels. DFX provided protection against the decline of ATP levels. This suggested the decline in ATP levels was iron-dependent or downstream from an iron-dependent event. In addition, the results indicated that loss of gluconeogenesis was not responsible for the decline in ATP levels as DFX protected the decline of ATP levels without protecting the loss of gluconeogenesis. In

consideration of the time frame for the decline in ATP levels, the decline of ATP levels must involve the iron-dependent pathway and be downstream from oxidative events as indicated by lipid peroxidation and changes in glutathione levels.

3.4.2 Component analysis

The experiments in this section were designed to determine if myoglobin as a whole or which components of myoglobin were responsible for the toxicities observed thus far. Several studies had suggested that the iron in the myoglobin was the determinant for toxicity. To simulate the iron component, ferric chloride ($FeCl_3$) was used. The ferric chloride was also pretreated with ascorbic acid to achieve ferrous (Fe^{2+}) iron. Incubation with iron in equimolar amounts to myoglobin produced a concentration-dependent increase in LDH release (Figure 23). The concentration-dependent LDH release with the $FeCl_3$ group was very similar to the myoglobin treated group, albeit the LDH release levels were slightly lower. The levels of LDH release were basically indistinguishable between the treatment groups with respect to concentrations. This might have suggested that complete release of iron (Fe^{2+}) from myoglobin occurred, thus causing toxicity that was indistinguishable from Fe^{2+}-induced LDH release. Zager and Foerder (1992) reported similar results with iron treatment, in that incubation of freshly isolated proximal tubule segments with iron (2mM Fe^{2+}/2mM Fe^{3+}) demonstrated a significant increase in LDH release above control. Although the levels of iron were two-fold higher than the concentration of iron in this study, the levels were equimolar to those levels achieved in Zager's glycerol-induced rhabdomyolysis model. Moreover, Zager (1993) concluded that there was a secondary release of iron

from intracellular stores in addition to myoglobin. Therefore, it cannot be stated that the iron-induced loss of viability in the iron-treated group approximated the release of iron from myoglobin, but suggested that the total iron component was largely responsible for loss of viability.

In contrast to the LDH release results, gluconeogenesis was not as diminished in the presence of iron treatment as with myoglobin treatment (Figure 24), suggesting that the free iron component of myoglobin had only a slight effect on the loss of gluconeogenesis. This agreed with the DFX experiments that indicated the loss of pyruvate-stimulated gluconeogenesis was not iron-dependent. Furthermore, this suggested that the heme and/or protein portion of the myoglobin were responsible for the loss of gluconeogenesis. As evidence that the probable cause was heme-related, the results reported by Nath *et al*. (1998) demonstrated a ten-fold increase of heme levels in the mitochondria following glycerol injection into rats.

In a further attempt to analyze myoglobin toxicity, a small amount of SnPP was used to inhibit HO, thus preventing the breakdown of myoglobin into protein, heme, and free iron. Previous studies have shown that SnPP conferred cytoprotection against myoglobin toxicity in HK-2 cells and isolated proximal tubular segment from glycerol-treated rats (Zager *et al*., 1995; Zager and Burkhart, 1997). Pretreatment of renal slices with SnPP provided slight protection against loss of viability (Figure 25). This suggested that the breakdown of myoglobin into components was not required to induce toxicity, possibly implicating the ferryl form of myoglobin as an accomplice to free iron in relation to lipid peroxidation and glutathione changes upstream from loss of membrane integrity. It is unlikely that the lack of protection against LDH release was due to

insufficient SnPP or low levels of expression of HO, because a dramatic result was noted in the gluconeogenesis measure of toxicity.

The effects of SnPP-pretreatment on myoglobin-induced changes in gluconeogenesis were different from the effects on LDH release. Gluconeogenesis was not compromised in renal slices pretreated with SnPP (Figure 26). This indicated that the breakdown of myoglobin into components was required to alter gluconeogenesis and thus cell function, furthermore, refuting the ferryl form of myoglobin as being responsible. The experiments with DFX supported this conclusion in that DFX did not confer protection against loss of gluconeogenesis (Figure 7); yet, DFX has the ability to detoxify the ferryl form of myoglobin (Turner *et al*., 1991). The protection afforded by SnPP also suggested that the heme or protein component of myoglobin could be responsible for loss of gluconeogenesis. Perhaps, intact myoglobin does not enter the mitochondria but the heme and/or protein does and thus causes the loss of gluconeogenesis.

As a final attempt to analyze the components of myoglobin resulting in toxicity, renal slices were incubated with protoporphyrin IX (PP9) in place of myoglobin to simulate the protoporphyrin component of myoglobin. However, at equimolar concentrations of 1mg/mL myoglobin, the PP9 would not remain in solution. SnPP was chosen as an alternative to PP9. In keeping with solubility parameters, concentrations of 0.057, 0.114, and 0.227 mM SnPP were used as these concentrations were equimolar to 1, 2, 4 mg/mL concentrations of myoglobin. Furthermore, the low and medium concentrations of SnPP represented 25% and 50% released heme from 4 mg/mL myoglobin by HO. Incubation of renal slices with SnPP resulted in a decrease of

LDH release (Figure 27). This was in direct contrast to the myoglobin-induced increase in LDH release. To eliminate the possibility of interference in the LDH assay, the sum of LDH in the media and tissue was calculated. The total LDH per gram tissue was not significantly different, indicating the results were not an artifact of the assay. In turn, the results suggested that the protoporphyrin portion of myoglobin was not responsible for the loss of viability. The protoporphyrin or tin appeared to have a protective effect. Nevertheless, the protoporphyrin portion of myoglobin is unlikely to be involved with the loss of membrane integrity.

Contrary to LDH release, the effect of SnPP on gluconeogenesis was toxic. Renal slices incubated with SnPP lost gluconeogenesis capacity in a concentration-dependent manner (Figure 28). This was similar to the myoglobin-induced loss of gluconeogenesis. The overall levels of gluconeogenesis were lower in the SnPP treated as compared to the myoglobin treated. This could be accounted for by different vehicles. The vehicle in myoglobin treatment was buffer, whereas in SnPP treatment, it was DMSO. DMSO is toxic yet can also serve as an antioxidant. Pretreatment with GSH demonstrated protection (Figure 9) against loss of gluconeogenesis, implicating radicals in the toxicity. Because DMSO has antioxidant properties, this could account for the difference in severity of the loss of gluconeogenesis in the SnPP treated as compared to the myoglobin treated. Taken with the slight iron-induced loss of gluconeogenesis, the experiments with SnPP implied that the heme portion of the myoglobin could be responsible for loss of gluconeogenesis. Again, this was supported by the report by Nath *et al*. (1998) that demonstrated a ten-fold increase of heme levels in the mitochondria following glycerol injection into rats.

In summary, the experiments with DFX suggested that gluconeogenesis was the only parameter of toxicity where changes were iron-independent. This also indicated that multiple factors contribute to the pathway of myoglobin toxicity with gluconeogenesis following an iron-independent course and both lipid peroxidation and glutathione alteration following an iron-dependent one. When placing this in the basic time frame of toxicity, it became apparent that loss of viability and alterations in ATP levels were downstream from the oxidative events. Furthermore, the component analysis revealed that free iron and possibly the ferryl form of myoglobin were responsible for the loss of viability; whereas, the heme and/or protoporphyrin portion of myoglobin was accountable for the compromise of gluconeogenesis.

DISCUSSION

Myoglobin was found to be toxic to rat renal cortical slices. This toxicity was concentration-dependent with toxic concentrations falling within previously reported clinical and experimental values. Moreover, the toxicity was time-dependent and in keeping with other reported experimental periods. The time frame for myoglobin toxicity consisted of early and late events. Loss of membrane integrity as measured by LDH release occurred at two hours in conjunction with a decline in ATP levels. Earlier events were oxidative in nature and were evidenced by lipid peroxidation, glutathione changes, and a compromise of gluconeogenesis capacity. In effect, loss of cell function and increases in oxidative damage preceded loss of viability by one hour.

The DFX data indicated a bifurcation of the early events with iron-independent gluconeogenesis changes residing on one fork while iron-dependent lipid peroxidation and glutathione changes occupied another. Moreover, DFX-induced protection of LDH release and alterations in ATP levels suggested these parameters were iron-dependent or downstream from iron-dependent events. Because gluconeogenesis remained drastically compromised regardless of DFX treatment while LDH release was prevented, gluconeogenesis appeared to not play a major role in the loss of viability and thus probably was not upstream from LDH release. Taken together, these results generate a basic time frame of mechanistic events (Figure 29).

Of the early events, gluconeogenesis was maintained in renal slices pretreated with GSH. Furthermore, substrate-stimulation of gluconeogenesis indicated the site of toxicity to be in the mitochondria. Together, these findings suggested that the loss of gluconeogenesis was free radical-induced in addition to being mitochondrial.

Pretreatment of renal slices with DFX indicated that radical generation was not iron-dependent. This finding eliminated the possibility of a hydroxyl radical contributing to myoglobin toxicity since hydroxyl radicals are propagated in an iron-dependent manner. In addition, concurrent treatment of the slices with myoglobin and DMTU resulted in a decrease of gluconeogenesis, supporting the conclusion that the radical species was not a hydroxyl radical. Because DFX can detoxify ferryl myoglobin and the associated globin radical, this form of myoglobin was also doubtfully responsible for the loss of gluconeogenesis capacity.

Oxygen radicals are the probable cause for the myoglobin-induced loss of gluconeogenesis. Zager (1996a) reported that myoglobin affected the respiratory chain. He postulated that the respiratory chain (site 3) was the principal source of free radicals that initiated heme-protein-associated lipid peroxidation. Later in 1997, Zager and Burkhart further reported that the treatment of HK-2 cells with myoglobin and Antimycin A resulted in a protection of viability as indicated by dye exclusion, thus indicating that the terminal respiratory chain was a mediator of both lipid peroxidation and myoglobin-induced cell death.

The SnPP data suggested that the heme portion of the myoglobin was the likely candidate for the loss of gluconeogenesis. Pretreatment of the renal slices with SnPP to block HO prior to incubation with myoglobin, produced a protection of gluconeogenesis. This indicated that the breakdown of myoglobin and thus release of the components was required to alter gluconeogenesis. In addition, incubation of the renal slices with iron or SnPP in place of myoglobin to simulate the iron and protoporphyrin portion of the protein resulted in a compromise of gluconeogenesis

capacity. Surprisingly, incubation with $FeCl_3$ did not produce the same effect on gluconeogenesis. Again, this suggested that either the heme or the protoporphyrin component of the myoglobin caused the loss of gluconeogenesis and not free iron. Reports in the literature indicate that high levels of heme occur in the mitochondria isolated from glycerol-injected rats (Nath *et al*., 1998).

The other early events, namely lipid peroxidation and glutathione changes, were greatly affected with the DFX pretreatment. Because these parameters indicated oxidative injury, the protection afforded by DFX gave rise to three potential mechanisms that could be responsible for initiating the damage. First, a hydroxyl radical could be involved in the myoglobin toxicity. However, this was refuted by the experiments with DMTU that revealed the lack of participation of a hydroxyl radical. A second possibility was the formation of the ferryl form of myoglobin and the associated globin radical. This mechanism cannot be ruled out without further study. Thirdly, free iron released from myoglobin could have been inducing toxicities. Although, it was probable that the ferryl form of myoglobin worked in conjunction with the free iron to instigate damage.

Lipid peroxidation and changes in glutathione preceded the later events of loss of membrane integrity and alterations in ATP levels. These later events appear to directly result from oxidative events. Everything that afforded protection of the oxidative events provided protection of loss of membrane integrity and alterations in ATP levels. Furthermore, a severe compromise of gluconeogenesis did not always lead to the occurrence of the later events of ATP decline and LDH release. For example, pretreatment of renal slices with DFX protected against myoglobin-induced LDH release without protecting loss of gluconeogenesis capacity.

In consideration of the relationship between LDH release and ATP changes, the decline in ATP levels seems to be caused by leakage. Two pieces of evidence supported this. The percentage of LDH release above control in the myoglobin-treated group was the same percentage of decline below control in ATP levels. Furthermore, when renal slices were incubated with pyruvate, the percent of LDH release over control with the 12 mg/mL myoglobin treatment was ~30%, as was the percentage of ATP decline in the 12 mg/mL myoglobin- treated versus control.

The protection of myoglobin-induced LDH release by pyruvate was investigated further. To eliminate the possibility that pyruvate was simply acting as a substrate for energy, the experiments were repeated using glucose in place of pyruvate. Because glucose did not provide the same protection as pyruvate, it was concluded that conversion to glucose was not the protective mechanism of pyruvate. Previous studies had reported increased levels of H_2O_2 in rhabdomyolysis. To insure that pyruvate was not acting to scavenge H_2O_2 and prevent hydroxyl radical formation, experiments with DMTU, were performed. DMTU failed to provide protection of LDH release, indicating that pyruvate was not acting to decrease hydroxyl radical formation.

Investigation of the other parameters of toxicity revealed that pyruvate lent protection to the oxidative events and those events downstream from oxidative events. A close evaluation of the pyruvate effect on glutathione levels exposed a possible explanation for pyruvate protection. In renal slices concomitantly treated with pyruvate and myoglobin, the total glutathione levels were increased significantly above the level measured in the untreated control group. In effect, the presence of pyruvate induced an increase in total glutathione levels, enabling the tissue to deal more efficiently with

oxidative stress. The pyruvate-induced increase in ATP levels would supplement the protective effect of increased total glutathione levels by providing sufficient ATP for the cycling of oxidized glutathione to reduced glutathione and for the fueling of repair systems.

Another part of this study addressed the role of myoglobin and its components that may be contributing to the toxicities. Incubation of renal slices with equimolar amounts of $FeCl_3$ as compared to myoglobin revealed that the iron portion of the myoglobin was involved mostly with loss of membrane integrity. Gluconeogenesis was compromised in the presence of iron but not to the extent of myoglobin-induced decrease in gluconeogenesis. Prior to incubation with myoglobin, pretreatment of renal slices with SnPP to inhibit HO led to a modest protection of gluconeogenesis without affecting LDH release much. This observation suggested that the breakdown of myoglobin was somewhat required to compromise gluconeogenesis but not membrane integrity. Furthermore, treatment of the renal slices with SnPP in place of myoglobin to simulate the heme portion of the protein resulted in decreased gluconeogenesis capacity. The effect of SnPP on LDH release was more difficult to interpret as it decreased LDH release below control group levels in a concentration-dependent manner. This effect may have been a result of the tin portion instead of the protoporphyrin. Regardless, SnPP affected gluconeogenesis without causing LDH release, implicating the heme component as the cause for loss of gluconeogenesis.

In summary, the mechanism of myoglobin toxicity appeared to involve iron-independent and dependent radical events that preceded loss of viability. The initial events of toxicity included heme-induced loss of gluconeogenesis and iron-dependent

oxidative damage. Furthermore, the protection afforded by pyruvate resulted from an overall increase in total glutathione and ATP levels.

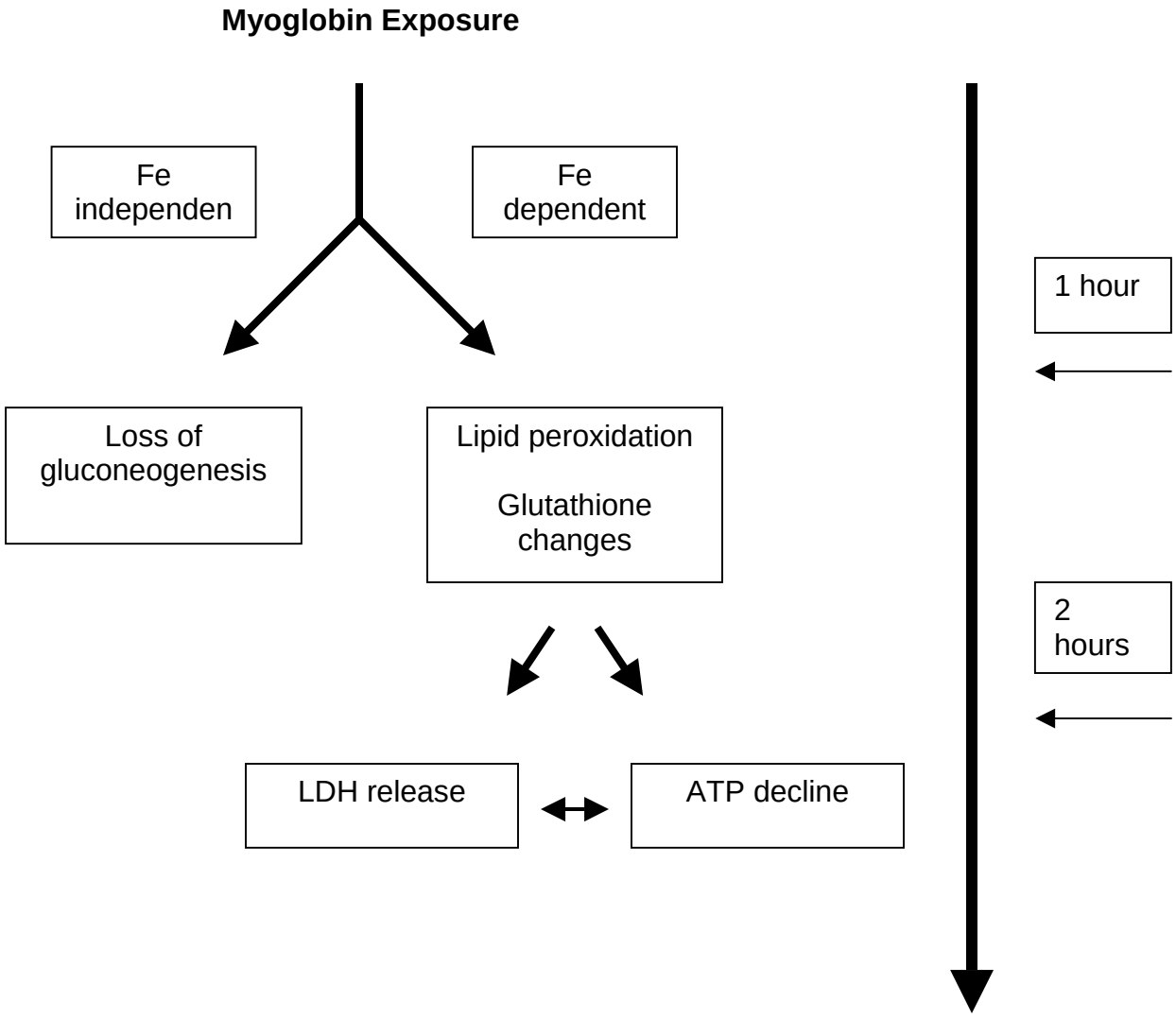


Figure 29. Time frame for mechanistic events of myoglobin toxicity.

FUTURE STUDIES

The current study addressed many aspects of myoglobin toxicity in the renal cortical slice model, which can be extrapolated to the pigment nephropathy component of the clinical manifestation of rhabdomyolysis. However, the current study also raises additional questions concerning myoglobin toxicity in this model. These questions, as well as future studies to address them, are discussed as follows.

This study indicates that free iron and the ferryl from of myoglobin are associated with the oxidative events of lipid peroxidation and alterations in glutathione levels. However, the specific contribution of each component remains to be elucidated. Future studies could directly investigate the role of free iron on lipid peroxidation and glutathione changes, to complement the data containing the effects on LDH release and gluconeogenesis. To dissect the role of ferryl myoglobin, experiments would be conducted at a lower pH. Previous literature reports that myoglobin-induced ARF is potentiated by acidosis (Heyman *et al*., 1997; Abassi *et al*., 1998; Holt and Moore, 2000). In addition, previous literature has reported that the ferryl from of myoglobin is more stable in an acidic environment and that at a higher pH the ferryl form becomes unstable and thus unable to induce lipid peroxidation (Moore *et al.,* 1998; Holt and Moore, 2000). The current study was performed in Krebs-Ringer buffer, pH 7.4. Additional studies would be performed at a lower pH and compared to those studies performed at pH 7.4. Hypothetically, during a two-hour incubation with myoglobin, the amount of lipid peroxidation should increase in the myoglobin treated tissue as compared to the control tissue at lower pH such as 6.0. The amount of change would

implicate the ferryl from of myoglobin and indicate the relative amount of participation in the overall toxicity.

To complete the component analysis, additional studies would be performed using SnPP in place of myoglobin in the incubations. To supplement the LDH release and gluconeogenesis data, lipid peroxidation and glutathione levels would be measured. Hypothetically, SnPP should not affect these measures to toxicity following incubation of renal slices with myoglobin. However, because these parameters indicate oxidative injury and because SnPP may possess anti-oxidative properties, the toxicities may be decreased making the interpretation of the data difficult.

The effects of pyruvate were interesting yet perplexing. Additional studies need to address how pyruvate induces an increase in total glutathione levels. Using BSO and BCNU, inhibitors of glutathione synthesis and redox cycling respectively, would indicate if the effect is synthesis or redox cycle related. Because pyruvate is present in both the cytosolic and mitochondrial compartments, other studies would address the cytosolic and mitochondrial glutathione independently to elucidate the relative location of the effect. Studies using other α-ketoacids such as oxaloacetate could indicate if the effect was specific to pyruvate or the result of a more general α-ketoacid effect.

APPENDIX 1: Detailed Methods

Krebs Ringer buffer

(126.4 mM NaCl; 6.0 mM Na_2HPO_4; 5.2 mM KCl; 1.3 mM $MgSO_4$; 1.3 mM $CaCl_2$)

Into approximately 800 mL of deionized distilled water, the following salts were added: 7.39 g NaCl; 852 mg Na_2HPO_4; 385 mg KCl; 318 mg $MgSO_4$; 149 mg $CaCl_2$. The pH was adjusted to 7.4 with HCl and/or KOH. The volume was brought to 1 L with deionized distilled water, followed by a final pH check.

Phosphate buffer for HPLC

To approximately 900 mL of deionized distilled water, 13.6 g KPO_4 was added. The pH was adjusted to 6.0 with HCl and KOH. The volume was brought to 1 L with deionized distilled water, followed by a final pH check. The buffer was filtered through a Millipore pre-filter (AP15, 47 mm).

Thiobarbituric acid (TBA)

TBA (167 mg) was dissolved in water and brought to 25 mL. The flask was wrapped in foil and warmed to ~90 ^{o}C.

Preparation of myoglobin

Horse skeletal muscle myoglobin (Sigma, M-0630) was used for all the studies. Various amounts of myoglobin (0, 60, 150, 180 mg) were dissolved in 5 mL Krebs-Ringer buffer. To each beaker, 100 μL of ascorbic acid (35.2 mg/mL water) was added. The beakers

were covered and sonicated for a minimum of 30 minutes. A color change from brown to red indicated successful reduction of the iron in the myoglobin.

Preparation of ferric chloride ($FeCl_3$)

Ferric chloride (Aldridge, I-88) was used for all $FeCl_3$ studies. To 5 mL of Krebs-Ringer buffer, 20 mg of $FeCl_3$ was added. Various amounts of this $FeCl_3$ solution (0, 231, 577, 690 μL) were brought to 5 mL with Krebs-Ringer buffer. To each beaker, 100 μL of ascorbic acid (35.2 mg/mL water) was added. The beakers were covered and sonicated for a minimum of 30 minutes.

Preparation of Sn(IV) Protoporphyrin IX (SnPP)

(For incubation studies with SnPP only)

Sn(IV) Protoporphyrin IX (Frontier Scientific; Porphyrin Products , Sn749-9) was used for all SnPP studies. Various amounts of SnPP (1.28 mg, 2.56 mg, 5.11 mg) were brought to 1 mL with DMSO. Flasks were vortexed on high for one minute followed by a sonication for a minimum of 30 minutes.

Preparation of Protoporphyrin IX (PP9)

Protoporphyrin IX (Sigma, P8293) was used for all PP9 studies. Various amounts of PP9 (3.83 mg, 9.60 mg, 11.63 mg) were brought to 1 mL with DMSO. Flasks were vortexed on high for one minute followed by a sonication for a minimum of 30 minutes.

Incubation of renal slices with SnPP

Following the rinse, renal slices (total weight range 50-100 mg) were placed in 3 mL oxygenated Krebs-Ringer buffer and allowed to warm for 10 minutes at 37°C under a 100% oxygen atmosphere and constant shaking (100 cycles/minute). Upon equilibration of the slices, 20 μL of (35.2 mg/mL water) ascorbic acid was added to each flask in addition to 100 μL of SnPP (final concentration 0-0.227 mM SnPP and 1.33 mM of ascorbic acid). The slices were incubated for 90 minutes at 37°C under a 100% oxygen atmosphere and constant shaking (100 cycles/minute). Directly following the 90-minute incubation, 100 μL of pyruvate (165 mg/5 mL water; 10 mM final) was added to stimulate gluconeogenesis. Upon completion of an additional 30-minute incubation, the media was decanted and the tissue was blotted and weighed. Toxicity was assessed as described.

Incubation of renal slices with $FeCl_3$

Following the rinse, renal slices (total weight range 50-100 mg) were placed in 2 mL oxygenated Krebs-Ringer buffer and allowed to warm for 10 minutes at 37°C under a 100% oxygen atmosphere and constant shaking (100 cycles/minute). Upon equilibration of the slices, a 1 mL aliquot of the $FeCl_3$ / ascorbic acid solution was added to the 2 mL of buffer containing the tissue slices (final concentration of 0-0.69 mM $FeCl_3$ and 1.33 mM of ascorbic acid). The slices were incubated for 90 minutes at 37°C under a 100% oxygen atmosphere and constant shaking (100 cycles/minute). Directly following the 90-minute incubation, 100 μL of pyruvate (165 mg/5 mL water; 10 mM final) was added to stimulate gluconeogenesis. Upon completion of an additional 30-

minute incubation, the media was decanted and the tissue was blotted and weighed. Toxicity was assessed as described.

Incubation of renal slices with PP9

Following the rinse, renal slices (total weight range 50-100 mg) were placed in 3 mL oxygenated Krebs-Ringer buffer and allowed to warm for 10 minutes at 37°C under a 100% oxygen atmosphere and constant shaking (100 cycles/minute). Upon equilibration of the slices, 20 μL of (35.2 mg/mLwater) ascorbic acid was added to each flask in addition to 100 μL of PP9 (final concentration 0-0.69 mM PP9 and 1.33 mM of ascorbic acid). The slices were incubated for 90 minutes at 37°C under a 100% oxygen atmosphere and constant shaking (100 cycles/minute). Directly following the 90-minute incubation, 100 μL of pyruvate (165 mg/5 mL water; 10 mM final) was added to stimulate gluconeogenesis. Upon completion of an additional 30-minute incubation, the media was decanted and the tissue was blotted and weighed. Parameters were assessed as described previously in Chapter 1 Materials and Methods.

Total glutathione levels

Following incubation, the tissues were rinsed in 3 mL of Krebs-Ringer buffer, blotted and weighed. Once weighed, the tissues were placed in 500 μL of 20% sulfosalycilic acid and homogenized followed by a rinse of the homogenizer shaft with an additional 500 μL of 20% sulfosalycilic acid. The mixture was centrifuged at 9000 g for 10 minutes at 4°C. The supernatant was decanted and saved. For total glutathione levels, 25 μL of supernatant was combined with 175 μL water and 700 μL of 0.3 mM NADPH. Following

a 10-minute incubation at 30°C, 100 μL of 6 mM DTNB and 100 μL of glutathione reductase (16 units/mL) were added. The samples were placed immediately into the spectrophotometer set at 412 nm and read at 0,15,30,60, and 90 seconds. Values were calculated using a standard curve of reduced glutathione.

Disulfide glutathione levels

(Tietze, 1969; Andersen, 1985)

Following incubation, the tissues were rinsed in 3 mL of Krebs-Ringer buffer, blotted and weighed. Once weighed, the tissues were placed in 500 μL of 20% sulfosalycilic acid and homogenized followed by a rinse of the homogenizer shaft with an additional 500 μL of 20% sulfosalycilic acid. The mixture was centrifuged at 9000 g for 10 minutes at 4°C. The supernatant was decanted and saved. For disulfide glutathione levels, 25 μL of supernatant was combined with 175 μL water. To this, 2 μL of triethylamine and 4 μL of 4-vinylpyridine were added and the mixture was incubated for 30 minutes at room temperature. Following the incubation, 700 μL of 0.3 mM NADPH was added with an additional 10-minute incubation at 30°C. After this incubation, 100 μL of 6 mM DTNB and 100 μL of glutathione reductase (16 units/mL) were added. The samples were placed immediately into the spectrophotometer set at 412 nm and read at 0,15,30,60, and 90 seconds. Values were calculated using a standard curve of disulfide glutathione.

Isolation of microsomes

Male Fischer 344 rats were anesthetized with ethyl ether and the livers were removed, wrapped in foil, and placed on ice. From each liver, approximately 4 g of tissue was weighed and placed into 4 mL of phosphate buffer (0.067 M, pH 7.4). The tissue was homogenized and the shaft was rinsed with an additional 1mL of phosphate buffer. The mixture was placed into an ice-cold 25 mL graduated cylinder. Phosphate buffer was used to adjust the volume to four times the recorded weight. The homogenate was centrifuged for 15 minutes at 10,000 g, 4°C. The supernatant was placed into a polycarbonate centrifuge tube, counterbalanced, and ultra-centrifuged for one hour at 100,000 g (40,000 rpm), 4°C. The supernatant was discarded and the glycogen pellet was removed. The remaining pellet was re-suspended with 2 mL phosphate buffer and kept on ice. A Bradford assay was performed to determine the protein content (Bradford, 1976). The protein concentration was adjusted to 4 mg/mL. Aliquots (250 μL) were stored at -70°C.

APPENDIX 2: List of Tables and Figures

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