Myoglobin toxicity in proximal human kidney cells: Roles of Fe, Ca^{2+}, H_2O_2, and terminal mitochondrial electron transport

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Myoglobin toxicity in proximal human kidney cells: Roles of Fe, Ca^{2+}, H_2O_2, and terminal mitochondrial electron transport. The purpose of this study was to gain direct insights into mechanisms by which myoglobin induces proximal tubular cell death. To avoid confounding systemic and hemodynamic influences, an *in vitro* model of myoglobin cytotoxicity was employed. Human proximal tubular (HK-2) cells were incubated with 10 mg/ml myoglobin, and after 24 hours the lethal cell injury was assessed (vital dye uptake; LDH release). The roles played by heme oxygenase (HO), cytochrome p450, free iron, intracellular Ca^{2+}, nitric oxide, H_2O_2, hydroxyl radical ($\cdot OH$), and mitochondrial electron transport were assessed. HO inhibition (Sn protoporphyrin) conferred almost complete protection against myoglobin cytotoxicity (92% vs. 22% cell viability). This benefit was fully reproduced by iron chelation therapy (deferoxamine). Conversely, divergent cytochrome p450 inhibitors (cimetidine, amino-benzotriazole, troleandomycin) were without effect. Catalase induced dose dependent cytoprotection, virtually complete, at a 5000 U/ml dose. Conversely, $\cdot OH$ scavengers (benzoate, DMTU, mannitol), xanthine oxidase inhibition (oxypurinol), superoxide dismutase, and manipulators of nitric oxide expression (L-NAME, L-arginine) were without effect. Intracellular (but not extracellular) calcium chelation (BAPTA-AM) caused ~50% reductions in myoglobin-induced cell death. The ability of Ca^{2+} (plus iron) to drive H_2O_2 production (phenol red assay) suggests one potential mechanism. Blockade of site 2 (antimycin) and site 3 (azide), but not site 1 (rotenone), mitochondrial electron transport significantly reduced myoglobin cytotoxicity. Inhibition of Na,K-ATPase driven respiration (ouabain) produced a similar protective effect. We conclude that: (1) HO-generated iron release initiates myoglobin toxicity in HK-2 cells; (2) myoglobin, rather than cytochrome p450, appears to be the more likely source of toxic iron release; (3) H_2O_2 generation, perhaps facilitated by intracellular Ca^{2+}/iron, appears to play a critical role; and (4) cellular respiration/terminal mitochondrial electron transport ultimately helps mediate myoglobin's cytotoxic effect. Formation of poorly characterized toxic iron/H_2O_2-based reactive intermediates at this site seems likely to be involved.

Rhabdomyolysis-induced acute renal failure (ARF) has been a subject of extensive laboratory investigation since Bywaters and Beall's classic descriptions of this syndrome following the London bombing raids of World War II [1]. These investigations have clearly demonstrated that this form of ARF has a multifactorial basis. Three major and interactive injury pathways have been noted [2]. First, renal vasoconstriction, a consequence of intravascular volume depletion and altered expression of vasoactive

compounds (such as nitric oxide, endothelin, tumor necrosis factor, adenosine, platelet activating factor [2–8]) markedly reduce renal blood flow (RBF). This directly decreases GFR [9] and it may also produce ischemic tubular damage [10]. Second, myoglobin precipitation within distal tubules produces cast formation, and possibly, intratubular obstruction [11–13]. Third, proximal tubular myoglobin uptake via endocytosis permits expression of its direct cytotoxic effects [14].

Multiple interrelationships between these three injury pathways exist and serve to amplify the evolving tissue damage [2]. Some examples include: renal vasoconstriction markedly potentiates myoglobin's cytotoxic effects [10, 15]; cast formation accentuates proximal tubular cell heme protein uptake, increasing the potential for direct cytotoxicity [14]; and if tubular cell death results, the necrotic debris provides additional substrate for cast formation, worsening tubular obstruction and filtration failure. The interdependency of these pathways is underscored by the fact that interference with just one of them (such as attenuation of vasoconstriction or cast formation) can completely block the development of ARF [16].

In recent years, most investigations in this area have focused on defining the mechanisms by which myoglobin exerts its cytotoxic effect. The approach generally taken has been to administer pharmacologic agents targeted against a specific injury pathway, and then assess whether the severity of myoglobinuric ARF has been affected. These investigations have produced a number of important insights. In sum, they suggest that much of myoglobin's cytotoxic effect stems from iron-driven hydroxyl radical ($\cdot OH$) generation via the Haber Weiss reaction [17, 18]. This conclusion stems from observations that iron chelator (deferoxamine) or $\cdot OH$ scavenger therapy (benzoate, dimethylthiourea) mitigates myohemoglobinuric ARF [17, 18]. However, given the multifactorial and interactive nature of *in vivo* myohemoglobinuric renal injury, as noted above, it is impossible to know whether these results stem from direct versus indirect drug effects on the proximal tubular epithelium. Furthermore, since the glomerular filtration barrier prevents delivery of large molecular weight probes to the proximal tubular epithelium (antioxidant enzymes such as catalase), it is impossible to fully probe injury pathways with *in vivo* experiments.

Given these limitations, *in vitro* studies of myoglobin cytotoxicity are required to further test specific hypotheses. To this end, our laboratory recently developed an *in vitro* model for studying myoglobin cytotoxicity [19, 20]. Rhabdomyolysis was induced in either rats or mice, and after allowing one to two hours for proximal tubular myoglobin uptake, proximal tubular segments

were isolated from the kidneys to allow *in vitro* study of rapidly evolving heme protein cytotoxicity. The principal conclusions from those studies were as follows: (a) iron is a critical determinant of myohemoglobinuric renal injury, as demonstrated by the fact that DFO confers a cytoprotective effect; (b) lipid peroxidation is driven by mitochondrial free radical generation, since sites 2 and 3 (but not site 1) mitochondrial respiratory chain inhibitors blocked lipid peroxidation (but not cell death); and (c) lipid peroxidation and lethal cell injury are not directly linked (since multiple agents can produce divergent changes in these two parameters). However, two potential experimental caveats need to be kept in mind when interpreting these data: first, tubule extraction produces isolation artifacts; and second, the heme-induced proximal tubular injury was well advanced at the time the tubules were studied in the *in vitro* setting (as evidenced by a rapid evolution of cell death). Thus, relatively late, rather than early, injury events were probed with this model system.

The present studies were designed to further explore mechanisms of myoglobin toxicity using a completely *ex vivo* system. By so doing, the limitations imposed by both whole animal experiments and isolated tubule systems can be avoided. To this end, we have recently observed that if myoglobin is added to cultured human proximal tubular (HK-2) cells in doses associated with *in vivo* nephrotoxicity, lethal injury evolves over a 24 hour period [21]. Thus, this model could complement prior *in vivo* and *in vitro* investigations in two important ways: (1) cell injury can be studied without confounding systemic, hemodynamic, and intraluminal events; and (2) delivery of pharmacologic probes can be delivered directly to the target of interest during the early, rather than the late injury phase. Using this approach, we have sought to better define the mechanisms by which myoglobin mediates cytotoxicity. In particular, the following issues were addressed: What is the source and nature of cytotoxic iron generated in response to tubular cell myoglobin loading? Which reactive oxygen metabolites help to mediate iron's cytotoxic effect? What is the functional impact of cellular/mitochondrial respiration on the evolution of myoglobin cytotoxicity (that is, does it directly contribute to cell death)? Finally, does intracellular Ca^{2+} participate in these injurous events? The results of those studies form the basis of this report.

Methods

Cell culture conditions

All experiments were performed using HK-2 cells, an immortalized proximal tubular cell line derived from a normal adult human kidney [22]. They were maintained in keratinocyte serum free medium (K-SFM; Gibco/BRL, Grand Island, NY, USA) to which was added 2 mM glutamine, 5 ng/ml epidermal growth factor, 40 µg/ml bovine pituitary extract, 25 U/ml penicillin, and 25 µg/ml streptomycin. Prior to experimentation, the cells, grown in T 75 Costar flasks (Costar, Cambridge, MA, USA), were trypsinized and transferred to 12- or 24-well Costar cluster plates. After ~24 hours of recovery, they were studied, as described below. Cell viability in each experiment was assessed by determining the % of cells which maintained vital dye exclusion (8 µg/ml ethidium bromide, counterstained with 2.3 µg/ml acridine orange, and India ink added to quench background fluorescence). One hundred cells in each of two to three fields were examined, and the mean was taken as the value for a given well. If a given test

agent exerted a significant protective effect, confirmation of that result was sought by second technique (% lactate dehydrogenase, LDH, release; discussed below).

Model of myoglobin cytotoxicity

Twenty-four hours after subculturing the HK-2 cells in cluster plates, they were subjected to a myoglobin challenge (10 mg/ml; compatible with concentrations observed uring *in vivo* models of heme protein induced ARF [21]). A 40 mg/ml stock solution of horse skeletal muscle myoglobin (Sigma M0630; lot # 55H7015) was prepared in Hank's balanced salt solution (HBSS; with Ca and Mg) by sonication, followed by passage through a 0.45 µM low protein binding filter. Since only metmyoglobin (Fe^{3+}) was commercially available, and since only reduced myoglobin (Fe^{2+}) was found to be cytotoxic (pilot data and [23]), 12 mM vitamin C (pH'd to 7.4 with NaOH) was added to the stock solution to effect a metmyoglobin → myoglobin conversion. After a one hour incubation at room temperature, the myoglobin was added to the HK-2 cells (final concentration, 10 mg/ml–3 mM vitamin C). They were then incubated under these conditions for 24 hours.

To test the influence of potential injury pathways, cells were subjected to the myoglobin challenge in the presence or absence of selected pharmacologic probes (described below). All test reagents were added simultaneously with the myoglobin challenge. To prove that these test reagents had no independent effect on cell viability, they were added to the cells only in the presence of K-SFM + vitamin C. Cells cultured only in the presence of K-SFM + vitamin C served as controls. [Note: vitamin C had first been shown to have no independent effect on cell viability]. Each pharmacologic agent was tested on ≥ 4 wells of cells on ≥ 3 different occasions.

Effect of heme oxygenase generated free iron

Previous *in vivo* and isolated tubule experiments of heme protein toxicity have suggested that heme oxygenase (HO), the enzyme that splits the porphyrin ring releasing free iron, may either protect against [19, 24] or help mediate heme protein toxicity [19]. The following experiment sought to clarify HO's impact using the present cell culture system. HK-2 cells were subjected to the myoglobin challenge in the absence or presence of an HO inhibitor (20 or 100 µM Sn protoporphyrin; Porphyrin Products, Logan, UT, USA). It had first been demonstrated that Sn protoporphyrin had no independent effect on cell viability in the absence of the myoglobin challenge. After completing the 24-hour myoglobin challenge, cell viability was determined by vital dye exclusion.

If HO affects the expression of myoglobin cytotoxicity by releasing iron from the porphyrin ring, it should be possible to reproduce Sn protoporphyrin's effect by iron chelation therapy. To make this determination, HK-2 cells were challenged with myoglobin in the presence or absence of deferoxamine (DFO; 2 mM). Twenty-four hours later the vital dye exclusion was determined.

Influence of cytochrome p450 inhibitors on the expression of myoglobin cytotoxicity

Recent evidence suggests that cytochrome p450-derived iron is a potentially critical determinant of hypoxic [25] as well as myohemoglobinuric renal injury [26]. These conclusions were

based on observations that cytochrome p450 inhibitors can confer cytoprotective effects [25, 26]. The goal of this experiment was to test whether myoglobin-induced cytotoxicity, as expressed in HK-2 cells, is also cytochrome p450 dependent. HK-2 cells were incubated with myoglobin either in the absence or presence of one of three different cytochrome p450 inhibitors: cimetidine (0.1, 0.2, 0.5 mM [25, 26]); aminobenzotriazole (10, 25, 50 μM [27, 28]); or troleandomycin (0.1, 0.25, 0.5 mM [29, 30]). These treatments/ dosages had first been shown to have no independent effect on HK-2 viability. Their impact on cell viability was determined 24 hours later by vital dye exclusion.

Impact of antioxidant interventions on the expression of myoglobin cytotoxicity

The following experiments were designed to identify those reactive oxygen species which participate in myoglobin's cytotoxic effect.

(1) Catalase. HK-2 cells were subjected to the myoglobin challenge in the absence or presence of 100, 1000, or 5000 U/ml of bovine liver catalase (C40; Sigma Chemicals). Twenty-four hours later, the percent of vital dye exclusion was determined. As a control for this experiment, additional cells were exposed to the myoglobin challenge in the presence of heat inactivated (100°C × 30 min) catalase to test whether catalase destruction eliminated the preparation's cytoprotective effect. To further prove the specificity of the catalase effect, an equivalent amount of protein to the 5000 U/ml catalase treatment (bovine serum albumin, BSA; 0.2 mg/ml) was added to the myoglobin challenge to assess whether cytoprotection would result.

(2) ·OH scavengers. ·OH scavengers have exerted inconstant cytoprotective effects in *in vivo* and in isolated tubule models of heme protein cytotoxicity [16, 18, 19, 31]. To further test ·OH's pathogenetic role in heme protein-induced tubular injury, HK-2 cells were challenged with myoglobin in the presence or absence of either dimethylthiourea (DMTU; 10, 100, or 1000 μM) or Na benzoate (5 mM, 1 mM, 500 μM). Since mannitol, a membrane impermeant ·OH scavenger, protects against *in vivo* myohemoglobinuric ARF (such as [31]), its influence on myoglobin-induced HK-2 cell injury was also assessed (5, 10, 20 mM). Each experiment had its own myoglobin-only control group. After 24 hours, cell viability was assessed. It had first been demonstrated that none of these agents in the doses employed had an independent effect on HK-2 viability.

(3) Glutathione. Glutathione (GSH) administration has been reported to confer striking protection against the glycerol model of myohemoglobinuric ARF [32]. However, GSH has paradoxically increased lipid peroxidation in isolated proximal tubule studies of heme protein cytotoxicity [19]. The following experiment sought to clarify GSH's impact on the evolution of myoglobin cytotoxicity. HK-2 cells were subjected to the myoglobin challenge in the presence or absence of 4 mM glutathione (which had no independent effect on HK-2 viability). Twenty-four hours later, vital dye exclusion was assessed.

(4) Agents directed against superoxide dismutase. The following experiments assessed whether superoxide generation, such as via xanthine oxidase activity, is critical to myoglobin-induced cytotoxicity. HK-2 cells were subjected to the myoglobin challenge under normal conditions or in the presence of: (1) a xanthine oxidase inhibitor (oxypurinol, 100, 1000, 5000 U/ml; solubilized in 0.1 N

NaOH [33]); or (2) superoxide dismutase (10, 250, 500 U/ml; from bovine erythrocytes; S-2515, Sigma). Vital dye exclusion was determined 24 hours later. These agents had first been demonstrated to have no independent effect on cell viability.

(5) Nitric oxide (NO) as a potential determinant of myoglobin toxicity. NO, a nitrogen-based free radical, has been reported to be a potentially important mediator of acute tubular damage [34]. Furthermore, increments and decrements in NO expression have been found to protect against and exacerbate *in vivo* myohemoglobinuric ARF, respectively [3]. The present experiments assessed whether agents that alter NO expression influence the *in vitro* expression of myoglobin cytotoxicity. HK-2 cells were subjected to the myoglobin challenge under normal conditions or in the presence of either L-NAME (an NO synthase inhibitor) or L arginine (the NO synthase substrate). Each agent was added in either 1 or 3 mM concentrations (shown to have no independent effect on HK-2 viability). Cell viability was assessed 24 hours later by vital dye exclusion.

Cellular respiration as a determinant of myoglobin cytotoxicity

The goal of the following experiment was to assess the impact mitochondrial respiration/Na,K-ATPase driven respiration on the evolution of myoglobin cytotoxicity. Suggesting a potential impact are our prior observations that mitochondrial electron transport is a critical determinant of lipid peroxidation in heme-loaded isolated tubular segments [20]. HK-2 cells were subjected to the myoglobin challenge under the following conditions: (1) combined site 1 + site 2 respiratory chain inhibition (rotenone + antimycin A addition, respectively, 7.5 μM of each); (2) rotenone alone (7.5 μM); (3) antimycin alone (7.5 μM); or (4) 5 mM Na azide, a site 3 respiratory chain inhibitor. In each instance, co-incubated cells exposed to myoglobin only, or to the mitochondrial inhibitors only, served as controls. Cell viability was assessed 24 hours later. [Note, it has previously been determined that glycolysis is sufficient to maintain normal HK-2 cell ATP concentrations under conditions of mitochondrial respiratory chain inhibition; hence, cell viability is maintained] [35].

Next, the influence of Na,K-ATPase driven respiration was assessed. HK-2 cells were incubated with myoglobin in the absence or presence of ouabain (0.1 or 0.5 mM) and cell viability was determined 24 hours later. These ouabain doses had first been shown to have no independent effect on HK-2 viability.

One potential mechanism by which alterations in energy production/consumption could influence the expression of myoglobin cytotoxicity would be via decreased endocytic transport. This could potentially limit cell myoglobin uptake. To test this possibility, the impact of rotenone/antimycin and ouabain therapy on endocytic transport was assessed by measuring Lucifer yellow uptake (a fluorescent marker of endocytosis [36, 37]). HK-2 cells (2 × 10^5 were cultured for 4 hr under control conditions or in the presence of either 1 mM ouabain, or antimycin + rotenone (7.5 μM each). Lucifer yellow was then added (10 mg/ml; Molecular Probes, Eugene, OR, USA). After completing an additional three hours incubation, the cells were washed × 4 with 4°C phosphate buffered saline, they were solubilized in 0.5 ml of 0.25% Triton X-100, and the fluorescence intensity of the cell extracts was determined with a spectrofluorometer (excitation and emission, 430 and 540 nm, respectively). Extracts prepared from cells in the absence of Lucifer yellow served as a blank.

Role of Ca^{2+} as a determinant of myoglobin toxicity

The following experiments tested the hypothesis that cellular Ca^{2+} overload, with secondary activation of phospholipase A_2, calpain, or endonuclease, are important determinants of myoglobin-induced cytotoxicity. Supporting this possibility are previous observations that Ca^{2+} overload can exacerbate oxidant tissue damage [38].

(a) Ca^{2+} chelation experiments. HK-2 cells were challenged with myoglobin in the presence of either EGTA (1 mM; > extracellular Ca^{2+} concentration of 0.63 mM in the myoglobin challenge) or in the presence of an intracellular Ca^{2+} chelator (BAPTA-AM; 20 μM; Molecular Probes). Separate control groups (myoglobin alone) were run for each. It had first been determined that these Ca^{2+} chelators exerted no effect on cell viability in the absence of the myoglobin challenge.

(b) PLA_2 inhibitor experiments. Cells were subjected to the myoglobin challenge under: (1) control conditions; (2) in the presence of an intracellular PLA_2 inhibitor (50 μM aristolochic acid, in DMSO; Biomol, Plymouth, PA, USA) [39, 40]; or (3) in the presence of a plasma membrane restricted PLA_2 inhibitor (100 μM dibucaine) [41]. Previous data indicated that these doses are effective in preventing another model of HK-2 cell injury (fluoride toxicity), and that neither inhibitor, as employed, had an independent effect on HK-2 viability [42].

(c) Calpain inhibitor experiments. Recent studies have suggested that calpain, the dominant intracellular neutral protease, can be an important determinant of Ca^{2+} initiated toxic and hypoxic cell death [43, 44]. To assess its potential role in myoglobin toxicity, HK-2 cells were exposed to the myoglobin challenge under control conditions or in the presence of one of the following calpain inhibitors: (1) calpain 1 inhibitor (3.75, 7.5, 15 μg/ml; Sigma); (2) E64 (5, 10, 20 μg/ml; Sigma [44]); or (3) CBZ (0.1, 0.5, 1 mM; Sigma [44]). Each was shown to have no independent effect on cell viability.

(d) Endonuclease inhibitor experiments. Endonuclease activation has recently been suggested as a potentially critical mediator of Ca^{2+}/free radical dependent cell death, as evidenced by the ability of endonuclease inhibitors (Evans blue, aurintricarboxylic acid) to confer protective effects [45–49]. Hence, HK-2 cells were challenged with myoglobin in the absence or presence of one of these two endonuclease inhibitors (Evans blue: 2, 5 μM; aurintricarboxylic acid, 5, 10 μM). Pilot data confirmed that neither agent had an independent effect on HK-2 viability.

Confirmation of cytoprotection by LDH release

HK-2 cells were cultured in 12 or 24 well plates and they were subjected to the myoglobin challenge either under control conditions or in the presence of those agents previously shown by vital dye exclusion to confer a cytoprotective effect (2 mM DFO, 100 μM Sn protoporphyrin, 5000 U/ml catalase, 20 μM BAPTA-AM, 7.5 μM antimycin A, 5 mM Na azide, or 0.1 mM ouabain). After 24 hours, % LDH release was determined [23]. The results were compared to those obtained with myoglobin treatment alone (each test agent had its own comparative group; $N \geq 3$ for each).

Assessment of iron and cytosolic Ca^{2+} loading on cell H_2O_2 production

The following experiment assessed whether free iron exposure and/or cytosolic Ca^{2+} loading increases renal tubular H_2O_2 generation. To this end, H_2O_2 production was gauged by the phenol red assay [50]. Pilot studies demonstrated that this assay lacked sufficient sensitivity to detect H_2O_2 production by HK-2 cells. Therefore, proximal tubular segments (PTS) were isolated from normal mouse kidneys [20] and used for this purpose. Each PTS preparation ($N = 9$) were divided into five 2.5 ml aliquots and they were incubated at 37°C under 95% O_2/5% CO_2 as follows: (1) control incubation for < two minutes (to establish baseline values); (2) 30 minute incubations under normal conditions; (3) 30 minute incubations in the presence of 100 μM $FeSO_4$; (4) 30 minute incubations in the presence of 35 μM A23187 Ca^{2+} ionophore; and (5) 30 minute incubations in the presence of both $FeSO_4$ and Ca^{2+} ionophore. Phenol red (264 μM) and horseradish peroxidase (HRP; 2.2 μM) were added at the start of each incubation. After completing the incubations, a sample (150 μl) was removed to determine % LDH release, and then 100 μl of 1 N NaOH was added to 2 ml of the remaining aliquots to quench the phenol red/H_2O_2/HRP reaction. The aliquots were centrifuged, and the increase in H_2O_2-driven oxidation of phenol red (absorbance at 610 nm) was assessed. H_2O_2 concentrations were calculated from a standard curve constructed by exogenous H_2O_2 addition to PTS buffer (0, 10, 20, 30, 40, and 50 μM H_2O_2). Values were expressed as nmol H_2O_2/mg tubule protein. To confirm that the assay was detecting H_2O_2, 5000 U/ml catalase was added to the tubules during control and $FeSO_4$ incubations (to confirm that reductions in H_2O_2 resulted).

Calculations and statistics

All values are presented as means ± 1 SEM. Comparisons between groups were performed by either unpaired or paired Student's *t*-test, as appropriate. If multiple comparisons were made, the Bonferroni correction was applied.

Results
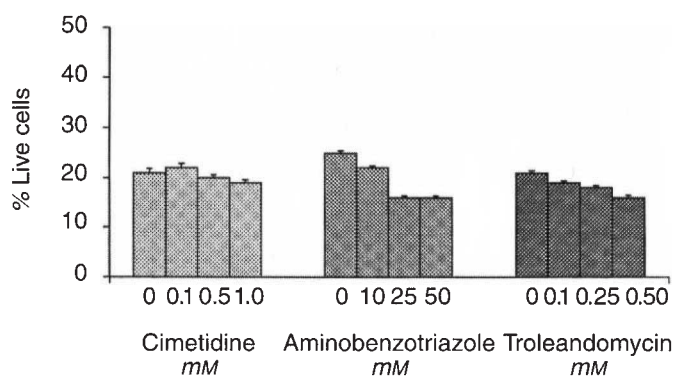
Myoglobin-induced cytotoxicity

The myoglobin challenge caused 60% to 85% loss of cell viability (vital dye uptake) by the completion of the 24-hour incubation period. In contrast, ≤ 5% vital dye uptake was observed when the cells were cultured only in the presence of vitamin C. The latter result was the same as that observed with no additions to the culture medium.

Heme oxygenase, iron, and cytochrome p450: Effects on myoglobin cytotoxicity

Heme oxygenase (HO) inhibition with Sn protoporphyrin induced dose dependent cytoprotection against myoglobin toxicity. At the 100 μM dose, essentially complete protection was observed (92 ± 2% vital dye exclusion; Fig. 1A). The importance of HO-driven iron release was further suggested by the fact that 2 mM DFO reproduced Sn protoporphyrin's cytoprotective effect (Fig. 1B). Conversely, cytochrome p450 did not appear to be a determinant of myoglobin cytotoxicity in HK-2 cells, since none of the cytochrome p450 inhibitors, at any of the concentrations, induced any cytoprotective effect (Fig. 2). If anything, a trend towards a worsening of myoglobin toxicity was apparent with each agent despite the fact that none had an independent toxic effect (Fig. 2).
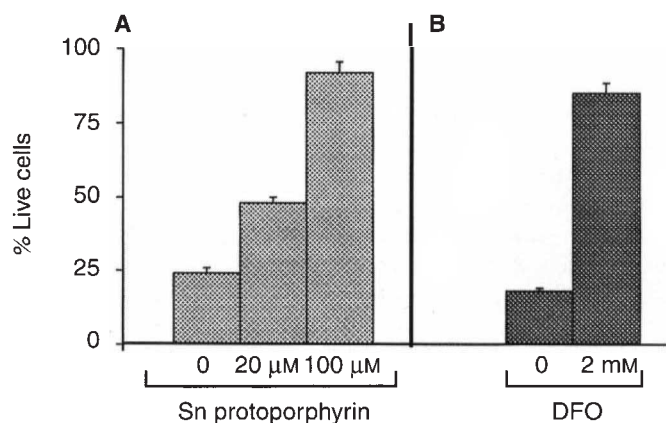
Fig. 1. *Heme oxygenase inhibition and iron chelation block myoglobin cytotoxicity.* Cells were incubated with myoglobin in the presence or absence of 20 or 100 μM Sn protoporphyrin (**A**) or DFO (**B**). Myoglobin caused ~80% loss of cell viability. Sn protoporphyrin (100 μM) and DFO each conferred essentially complete protection against myoglobin-induced cell death. 0 is myoglobin without any test agent; % live cells are by vital dye exclusion. $P < 0.00001$.

Fig. 2. *Cytochrome p450 inhibitors do not block myoglobin cytotoxicity.* Addition of either cimetidine, aminobenzotriazole, or troleandomycin (each in 3 different doses) failed to reduce the extent of myoglobin-induced cell death (vital dye uptake). 0 is myoglobin without any test agent; % live cells are by vital dye exclusion.

Impact of antioxidant strategies on myoglobin cytotoxicity

Neither DMTU, benzoate, nor mannitol induced any cytoprotection (Fig. 3 A, B). (The benzoate data were comparable to those obtained with mannitol, and for simplicity's sake, they are not depicted). Furthermore, neither superoxide dismutase (SOD; Fig. 3C) nor oxypurinol altered the extent of myoglobin cytotoxicity (Fig. 3D).

Manipulations designed to alter NO production had no influence on myoglobin's cytotoxic effect (data not depicted). Vital dye exclusion following myoglobin addition with either 0, 1, or 3 mM L-NAME were 26 $\pm$ 1%, 20 $\pm$ 1% and 23 $\pm$ 2%, respectively. In the presence of either 0, 1, or 3 mM L-arginine, 23 $\pm$ 1%, 19 $\pm$ 1%, and 24 $\pm$ 2% vital dye exclusions were observed. These data seemingly dissociated NO production from myoglobin's cytotoxic effect.

In contrast to the above, catalase induced profound dose-dependent cytoprotection, virtually eliminating myoglobin cytotoxicity at the 5000 U/ml dosage (91 $\pm$ 1% vital dye exclusion; Fig.

4). This protective influence was not a nonspecific consequence of protein addition (such as competition for myoglobin endocytic uptake), since the addition of an equivalent amount of bovine serum albumin (BSA) had no protective effect (with and without BSA, 20 $\pm$ 3% and 25 $\pm$ 4% vital dye exclusion, respectively). Heat inactivation of the catalase completely destroyed its cytoprotective effect (Fig. 4).

Despite the fact that the above data suggested that H_2O_2 is a critical determinant of myoglobin cytotoxicity, provision of exogenous 4 mM glutathione worsened the extent of myoglobin-induced cell death (Fig. 4). This was despite the fact that GSH exerted no independent cytotoxicity ($>$ 95% vital dye exclusion without myoglobin present).

Influence of mitochondrial/Na,K-ATPase driven respiration

As depicted in Figure 5 mitochondrial respiratory blockade with combined rotenone and antimycin therapy (R + A) significantly attenuated the expression of myoglobin cytotoxicity (46 $\pm$ 2% vs. 24 $\pm$ 1% vital dye exclusion). This protection was due to site 2, not site 1 inhibition since antimycin alone, but not rotenone alone, reproduced this result (Fig. 5). Site 3 respiratory blockade with Na azide was also protective (Fig. 5), indicating the critical role played by the terminal respiratory chain in the development of myoglobin cytotoxicity. Protection with azide was particularly noteworthy, given the fact that azide inhibits catalase (which would be expected to worsen myoglobin toxicity). When applied to HK-2 cells in the absence of myoglobin, neither antimycin, rotenone, nor azide caused substantial cell death (88 to 98% vital dye exclusion; due to the fact that glycolysis is sufficient to maintain HK-2 cell ATP concentrations under conditions of mitochondrial respiratory blockade [35]).

Ouabain also induced dramatic protection against myoglobin cytotoxicity (72 $\pm$ 4% vital dye exclusion at the 0.1 mM dosage; Fig. 6). This further indicates the critical role for cellular/mitochondrial respiration in the expression of myoglobin's cytotoxic effect.

The protection afforded by ouabain and mitochondrial respiratory blockade could not simply be ascribed to inhibition of endocytic transport. This is because these interventions tended to increase, rather than decrease, Lucifer yellow uptake (28 $\pm$ 6% and 97 $\pm$ 12% increments over control values with ouabain and mitochondrial inhibition, respectively; each $P < 0.005$).

Influence of Ca^{2+} as a determinant of myoglobin cytotoxicity

As shown in Figure 7, extracellular Ca^{2+} chelation with EGTA conferred no cytoprotection despite being used in a dose that has previously been demonstrated to block a known model of Ca^{2+} dependent HK-2 cell death [42]. Conversely, intracellular Ca^{2+} chelation with BAPTA-AM eliminated ~50% of myoglobin's cytotoxic effect (Fig. 7). The relative specificity of this result was indicated by the facts that: (1) in a different model of toxin-induced HK-2 injury, BAPTA-AM had no protective effect [42]; and (2) BAPTA-AM does not act as an iron chelator [46].

Representative results obtained with inhibitors of Ca^{2+} dependent processes are presented in Figure 8. The data obtained with differing doses of each were quite similar, and for the sake of simplicity, results obtained with only one concentration of each are depicted. Neither of the PLA_2 inhibitors (dibucaine, aristolochic acid) conferred a cytoprotective effect despite being used in
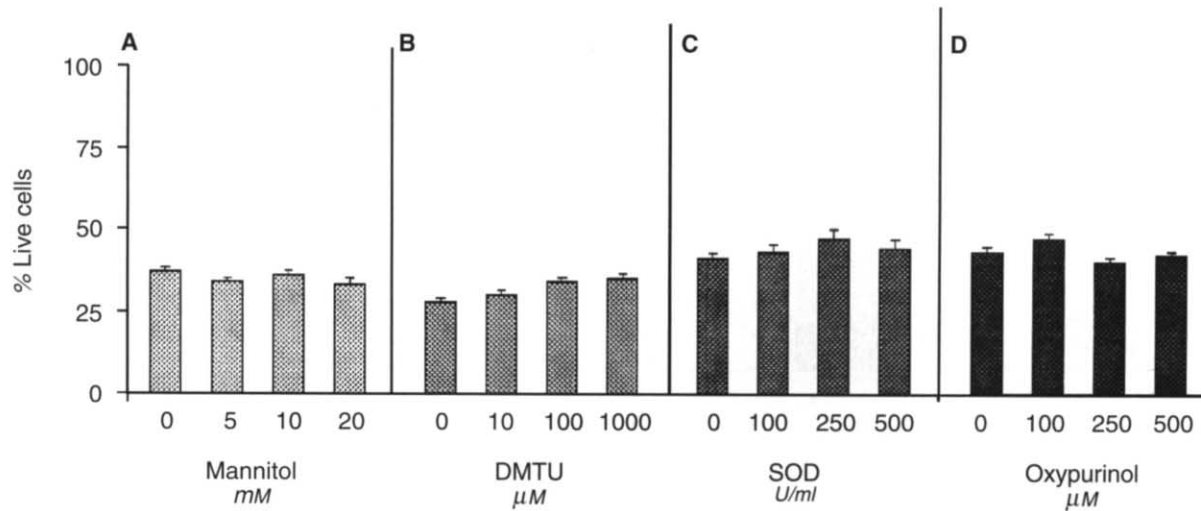
Fig. 3. *Hydroxyl radical scavengers, superoxide dismutase, and a xanthine oxidase inhibitor each fail to mitigate myoglobin's cytotoxic effect.* Membrane impermeable (mannitol) or permeable (dimethylthiourea, DMTU) ·OH scavengers failed to confer cytoprotection. (Not depicted are the Na benzoate data which were essentially identical to the mannitol results). Superoxide dismutase (SOD) and oxypurinol (an irreversible xanthine oxidase inhibitor) also exerted no protective effect. 0 is myoglobin without any test agent; % live cells are by vital dye exclusion.
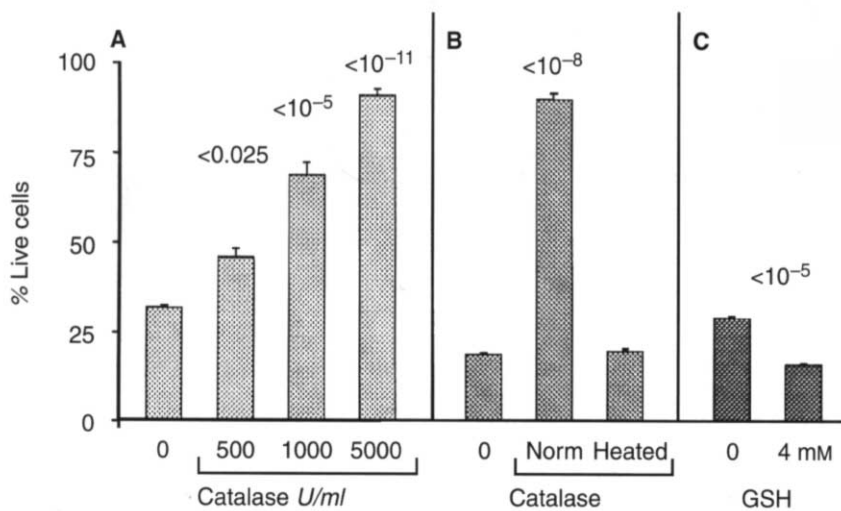
Fig. 4. *Catalase blocks, and GSH exacerbates, myoglobin-induced cytotoxicity.* **A.** Increasing doses of catalase induced increasing cytoprotection against myoglobin toxicity (vital dye uptake). At the 5000 U/ml dose, essentially complete protection was observed. 0 is myoglobin/no other treatment. **B.** If the 5000 U/ml catalase dose (Norm, normal) was applied, marked cytoprotection was, once again, observed. Heating catalase to 100°C × 30 minutes completely destroyed this cytoprotective effect, consistent with protection being due to catalase, rather than a contaminant. **C.** Addition of 4 mM GSH significantly worsened myoglobin-induced cell death. Not shown, GSH addition to cells in the absence of myoglobin induced no cell death (% live cells are by vital dye exclusion).

doses previously shown to be effective against a Ca^{2+}/PLA$_2$-dependent model of HK-2 cytotoxicity [42]. Although one protease inhibitor (CBZ) did confer significant protection, two other inhibitors (calpain 1 inhibitor; E64) failed to reproduce this result (suggesting that CBZ's effect was not specifically mediated by protease inhibition). Finally, neither endonuclease inhibitor (Evans Blue, EB; aurintricarboxylic acid, ATC) had a protective effect; in fact, the latter approximately doubled the extent of myoglobin-induced cell death despite being used in non overtly toxic concentrations; 10 μM results depicted in Fig. 8). Thus, endonuclease activation does not appear to be a critical determinant of myoglobin's toxic effect on HK-2 cells.

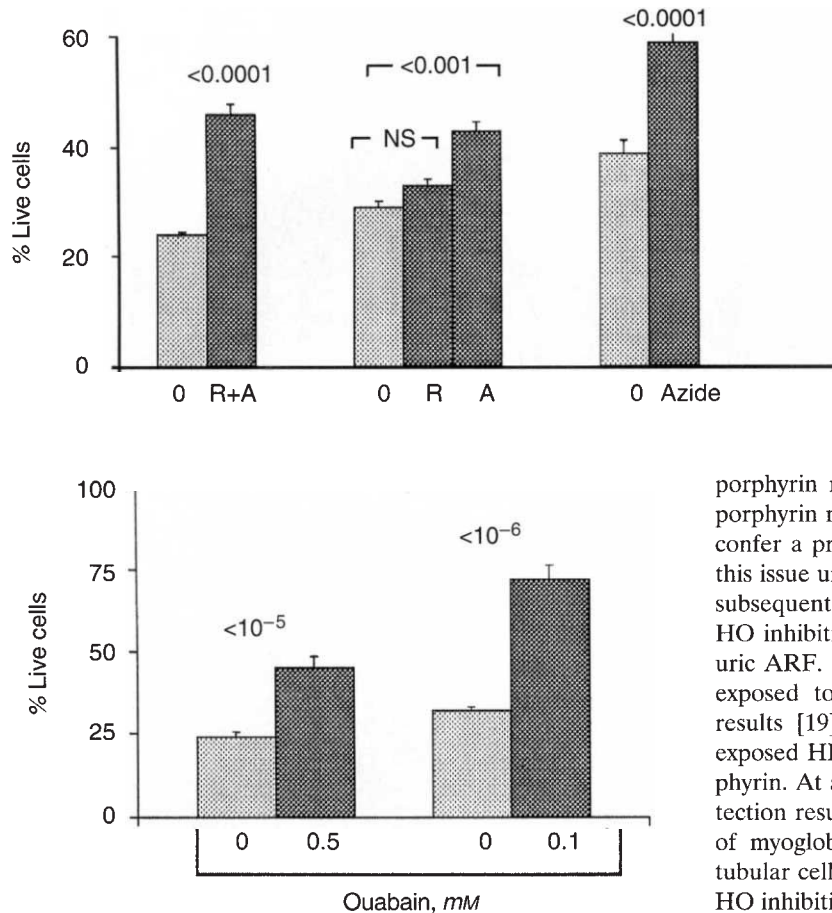### Confirmation of protection by LDH release

If an intervention was found to be protective by vital dye exclusion, that protection was confirmed by statistically significant reductions in % LDH release (Fig. 9).

Effects of iron and cytosolic Ca^{2+} loading on tubule H_2O_2 generation

At time zero (baseline), barely detectable H_2O_2 was detected in the PTS suspension (Fig. 10). After completing a 30-minute control incubation, 5.2 ± 0.8 nmol H_2O_2 production/mg protein was detected (Fig. 10). The addition of $FeSO_4$ and Ca^{2+} ionophore each caused significant increments in H_2O_2 generation, and when these two challenges were present together, fully additive (but not synergistic) H_2O_2 production resulted. The % LDH release from these challenges were as follows: control conditions, $12 \pm 1\%$; $FeSO_4$ challenge, $12 \pm 1\%$; Ca^{2+} ionophore, $18 \pm 2\%$; $FeSO_4 + Ca^{2+}$ ionophore, $20 \pm 3\%$. Only the combined $FeSO_4$ + ionophore challenge caused a statistically significant rise in LDH release (< 0.025).

Pilot data demonstrated that $FeSO_4$ addition to tubule buffer (that is, without tubules present) caused no H_2O_2 generation,

Fig. 5. *Site 2 (antimycin) and site 3 (azide), but not site 1 (rotenone) mitochondrial respiratory chain blockade significantly attenuate myoglobin cytotoxicity.* Addition of rotenone + antimycin A (R + A) approximately doubled the number of viable cells after completing the myoglobin challenge (left). This was due to antimycin (A), not rotenone (R), since antimycin alone, but not rotenone alone, induced protection. Na azide also conferred significant protection against myoglobin toxicity, further indicating the importance of the terminal portion of the mitochondrial electron transport chain to the mediation of myoglobin cytotoxicity. 0 is myoglobin with no other treatment; % live cells are by vital dye exclusion.

Fig. 6. *Na,K-ATPase inhibition confers protection against myoglobin toxicity.* Addition of either 0.1 or 0.5 mM ouabain significantly attenuated myoglobin toxicity. 0 is myoglobin/no other treatment; % live cells are by vital dye exclusion.

indicating that the source of H_2O_2 was the tubules. The specificity of the assay for H_2O_2 was confirmed by the fact that catalase addition to the tubules under control conditions and during the $FeSO_4$ challenge caused approximately 50% reductions in the detectable H_2O_2 generation.
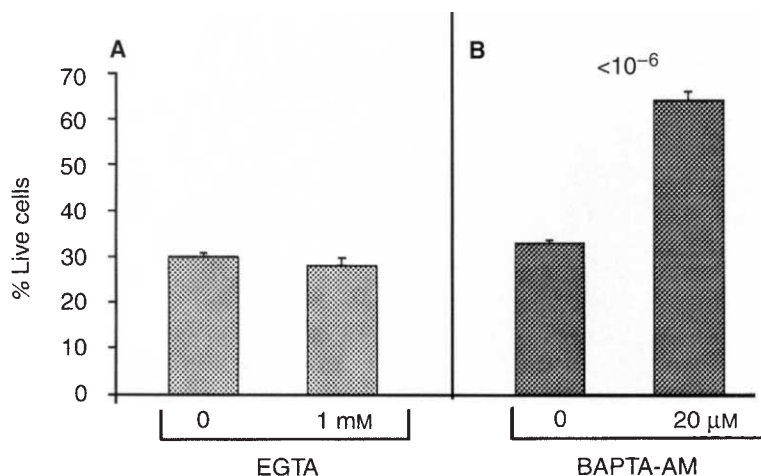
Discussion

The multifactorial nature of rhabdomyolysis-associated ARF complicates dissection of specific mechanisms by which myoglobin exerts its direct cytotoxic effect. While important clues can be gained by applying pharmacologic probes to *in vivo* experiments, the data obtained must be considered indirect. This is because any particular agent theoretically can alter tubular damage not by a primary action, but rather via an additional interposed event (such as changes in muscle injury, cytokine generation, cast formation, or intrarenal hemodynamics). Therefore, hypotheses derived from *in vivo* studies require confirmation by *in vitro* testing. The present study was designed to help achieve this objective.

The first specific goal of this study was to probe the role of heme oxygenase (HO) in the initiation of myoglobin's cytotoxic effect. If myoglobin toxicity were dependent on an intact porphyrin ring, HO inhibition should worsen myoglobin cytotoxicity by preserving

porphyrin ring integrity. Alternatively, if iron release from the porphyrin ring were critical to cytotoxicity, HO inhibition should confer a protective effect. Previously published studies have left this issue unresolved. For example, data from Nath et al [24] and subsequently confirmed in our laboratory [19] indicate that *in vivo* HO inhibition with Sn protoporphyrin worsens myohemoglobin-uric ARF. However, when myoglobin-laden isolated tubules are exposed to Sn protoporphyrin, a modicum of cytoprotection results [19]. In an attempt to resolve this paradox, we have exposed HK-2 cells to myoglobin with and without Sn protopor-phyrin. At a 100 μM concentration, essentially complete cytopro-tection resulted. Clearly then, HO is essential for the expression of myoglobin cytotoxicity. Since free iron is directly toxic to tubular cells [23], and since DFO fully reproduced the benefit of HO inhibition, it is highly likely that HO exerts its adverse impact by releasing toxic iron from the porphyrin ring. Only speculation is possible as to why HO inhibition paradoxically worsens *in vivo* myohemoglobinuric ARF. However, potential adverse systemic effects (such as a worsening of hemodynamics) could override a benefit expressed directly at the proximal tubular cell level [19].

The next goal of this study was to test whether the cytochrome p450 system is a second enzymatic determinant of myoglobin cytotoxicity. In this regard, Paller and Jacob have suggested that cytochrome p450 can serve as a labile source of intracellular iron, and that once released, it helps mediate hypoxic tubular cell injury [25]. Of direct relevance to the current study, Baliga et al recently reported that cytochrome p450 inhibitors (such as cimetidine) can mitigate the glycerol model of myohemoglobinuric ARF [26]. This led these workers to speculate that cytochrome p450, rather than myoglobin, may be the more important source of toxic iron release. Given the confounding variables inherent to *in vivo* experiments, we explored this hypothesis further by using the present cell culture system. Despite using three different cyto-chrome p450 inhibitors, including cimetidine, no cytoprotection resulted. There are a number of possible explanations for the seeming discrepancy between these data and those of Baliga et al. First, it could simply be that there is a down regulation of cytochrome p450 expression in cultured, compared to *in vivo*, tubular cells; if so, then a protective effect with cytochrome p450 inhibitors might only be observed in the *in vivo* state. Second, cytochrome p450 inhibitors might have affected *in vivo* myohemo-globinuric ARF [26] via a systemic, hemodynamic, or intraluminal effect, rather than by altering injury directly at the tubular level.

Fig. 7. *Intracellular, but not extracellular, Ca^{2+} chelation lessens myoglobin toxicity.* **A**. Addition of an extracellular Ca^{2+} chelator, EGTA, had no impact on the extent of myoglobin-induced cell death. **B**. Conversely, BAPTA-AM, an intracellular Ca^{2+} chelator, approximately doubled the number of cells which survived the myoglobin challenge. 0 is myoglobin/no other treatment; % live cells are by vital dye exclusion.
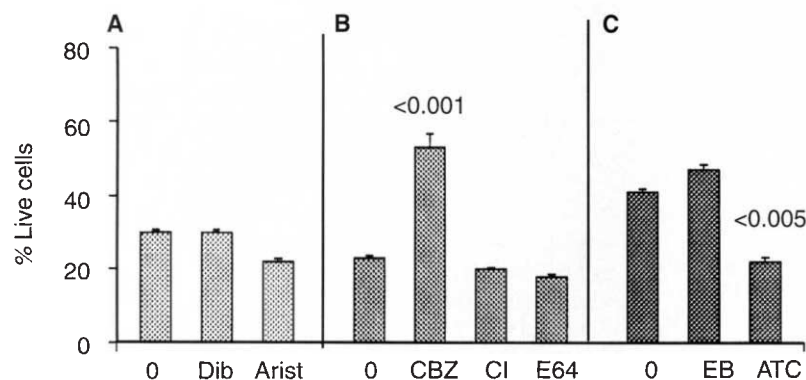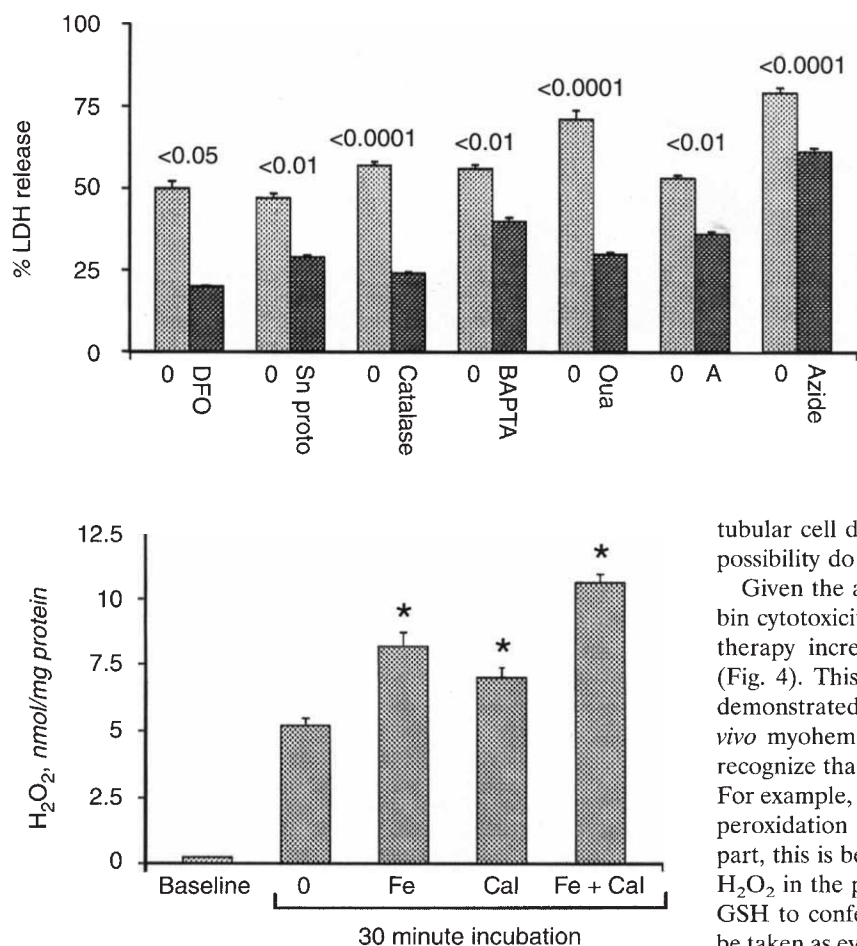

Fig. 8. *Inhibitors of (A) PLA_2 (B) calpain, and (C) endonuclease do not block myoglobin toxicity.* To probe potential pathways by which intracellular Ca^{2+} chelation might confer protection, the role of Ca^{2+} dependent enzymes on the expression of myoglobin toxicity was assessed. Neither of two PLA_2 inhibitors (100 μM dibucaine, dib; 50 μM aristolochic acid, arist), conferred protection. Although one calpain inhibitor (0.5 mM CBZ) was protective, this effect could not be reproduced by two other protease inhibitors (7.5 μM calpain 1 inhibitor, CI; or 20 μM E64). This suggests that CBZ's effect was probably not specifically mediated by protease inhibition. Neither of two endonuclease inhibitors (5 μM Evans blue, EB; 10 μM aurintricarboxylic acid, ATC) conferred protection; in fact, ATC worsened myoglobin toxicity without exerting an independent cytotoxic effect (data not shown). In summary, these data suggest that PLA_2, calpain, and endonuclease are not critical determinants of myoglobin-induced HK-2 cell death (% live cells are by vital dye exclusion).

Third, differing results could be due to the fact that the present study and Baliga's employed human and rat kidney cells, respectively. In this regard, marked differences in levels and isoforms of cytochrome p450 enzymes exist amongst different animal species. Whatever the exact explanation, one point seems clear: cytochrome p450 inhibitors do not necessarily block the expression of heme protein toxicity directly at the proximal tubular level.

Since a near constant correlate of myohemoglobinuric injury has been oxidative tissue damage, workers in this area have attempted to identify the responsible reactive oxygen metabolite(s). ·OH, generated from H_2O_2 in the presence of "catalytic" iron (that is, via the Haber Weiss reaction), has been most widely implicated. This is because OH scavengers (such as DMTU and mannitol) and an iron chelator (DFO) have been reported to mitigate *in vivo* myohemoglobinuric ARF. However, inconsistent results have been obtained, possibly because some antioxidants, such as DMTU, can exert potent systemic effects. That some critical probes of the Haber Weiss reaction (such as catalase) gain

little tubular access due to minimal glomerular filtration further complicates exploration of this issue. Hence, we have explored the impact of specific antioxidant strategies using the present cell culture system. No evidence for ·OH mediated damage could be obtained, since neither DMTU, benzoate, nor mannitol conferred a protective effect. While a negative result with mannitol could have been predicted because of limited intracellular access, DMTU and benzoate do penetrate cells, and still no protection resulted. This negative result, plus the inability of ·OH scavengers to block tubular injury induced by exogenous $FeSO_4$ [23], seemingly dissociate iron-induced tubular injury from ·OH production. Xanthine oxidase-driven superoxide has also been implicated as a mediator of acute tubular damage [reviewed in 33]. However, that neither oxypurinol nor SOD were protective helps acquit xanthine oxidase-driven superoxide production as a mediator of myoglobin toxicity. NO is a third reactive oxygen metabolite that purportedly causes tubular damage. However, neither NO inhibition (with L-NAME) nor L-arginine supplementation impacted on the extent of myoglobin-induced cell death. This suggests that the ability

Fig. 9. *Confirmation of cytoprotection by % LDH release.* Each agent that was found to protect against myoglobin toxicity, as assessed by vital dye exclusion, was also shown to be protective using a second marker of cell viability (reduction in % LDH release). Abbreviations are: DFO, 2 mM deferoxamine; Sn proto, 100 μM Sn protoporphyrin; oua, 0.1 mM ouabain; BAPTA-AM, 20 μM; A, 7.5 μM antimycin A; azide, 5 mM Na azide.

Fig. 10. *Free iron and cell Ca^{2+} loading each stimulate renal tubular H_2O_2 production.* Normal mouse proximal tubular segments were subjected to sublethal $FeSO_4$ (Fe; 100 μM) and Ca^{2+} ionophore (CaI; 35 μM A23187) challenges and H_2O_2 production was assessed by the phenol red technique at baseline and over the course of a 30-minute incubation period. In all instances, H_2O_2 production was observed (rise over baseline values), and the increment was significantly higher in the presence of Fe and CaI (*$P <$ 0.05). The $FeSO_4$ and ionophore-induced increments were fully additive (Fe + CaI).

of these two agents to alter *in vivo* myohemoglobinuric ARF stems from NO's hemodynamic actions, rather than from any direct tubular cell effect [3].

In striking contrast to the above, catalase was able to completely block myoglobin's cytotoxicity. To our knowledge, this is the first demonstration of this action, and it strongly suggests that H_2O_2 is a critical determinant myoglobin-initiated cell death. One potential mechanism by which H_2O_2 could induce lethal injury would be by fueling ·OH production via the Haber Weiss reaction. However, as discussed above, this seems unlikely. A more likely mechanism may be that H_2O_2, which liberates iron from heme proteins (such as in [51]), acts in concert with HO to produce critical free iron overload, and hence, direct iron-mediated toxicity [23]. Finally, since H_2O_2 and free iron can interact to form highly toxic, but poorly defined, reactive intermediates (other than ·OH [52]), a direct H_2O_2-iron derived moiety might mediate

tubular cell death. Unfortunately, specific probes to explore this possibility do not currently exist.

Given the apparent critical role of H_2O_2 in mediating myoglobin cytotoxicity, it may seem paradoxical that supplemental GSH therapy increased the extent of myoglobin-induced cell death (Fig. 4). This is particularly true since it has been convincingly demonstrated that systemic GSH administration can attenuate *in vivo* myohemoglobinuric ARF [32]. However, it is important to recognize that GSH, can exert pro- as well as anti-oxidant effects. For example, we have previously noted that GSH can worsen lipid peroxidation in heme loaded isolated tubular segments [19]. In part, this is because its constituent amino acid cysteine generates H_2O_2 in the presence of catalytic iron [53, 54]. Thus, a failure of GSH to confer protection against myoglobin toxicity should not be taken as evidence against H_2O_2 as an important determinant of heme toxicity. Rather, it may actually support this hypothesis [54].

It has previously been reported that increased H_2O_2 generation occurs during the course of the glycerol model of myohemoglobinuric ARF [55]. However, the reason for this has remained unknown. To this end, we tested the hypothesis that free iron, such as liberated by HO, directly stimulates H_2O_2 production. To address this possibility, $FeSO_4$ was added to isolated proximal tubules and increased tubule H_2O_2 generation resulted, as assessed over a 30 minute period. Since intracellular Ca^{2+} loading was also identified as a critical modulator of myoglobin toxicity (BAPTA-mediated protection), the impact of cell Ca^{2+} loading on H_2O_2 production was also assessed. Shortly after A23187 ionophore addition, increased H_2O_2 resulted. Given the diverse effects of cell Ca^{2+} loading on cell homeostasis, it is impossible to directly link this increased H_2O_2 production to Ca^{2+} mediated toxicity. However, no evidence was obtained to support other Ca^{2+} dependent processes (PLA$_2$, calpain, endonuclease activation) as being involved. Thus, Ca^{2+} driven H_2O_2/free radical generation remains a tenable hypothesis.

In a recent study, we demonstrated that the terminal portion of the mitochondrial electron transport system is a critical source of reactive oxygen metabolites during the course of myohemoglobinuria [20]. This conclusion was based on observations that addition of site 2 or site 3, but not site 1, mitochondrial respiratory chain inhibitors to heme laden isolated tubules (obtained from myohemoglobinuric mice) completely blocked *in vitro* malondialdehyde formation. However, the functional significance of this blockade

(that is, would it attenuate lethal cell injury?) could not be assessed. This was because mitochondrial inhibition in isolated tubules, and the resulting ATP depletion, rapidly induced cell death. Since HK-2 cells are not dependent on mitochondrial respiration to maintain ATP levels, and hence cell viability (that is, glycolysis is sufficient), they provided a unique opportunity to directly assess the contribution of mitochondrial electron transport to the evolution of myoglobin cytotoxicity. As depicted in Figure 8, addition of site 2 or site 3, but not site 1, respiratory chain inhibitors significantly attenuated myoglobin's cytotoxic effect. Therefore, it is clear that the terminal mitochondrial respiratory chain is not just a determinant of lipid peroxidation [20], it is also a critical mediator of myoglobin-induced cell death. To our knowledge, this is the first evidence that indicates this mechanistic relationship.

To further pursue the theory that cellular respiration is a key determinant of myoglobin toxicity, the impact of a more physiologically relevant manipulation of cell respiration was assessed: Na,K-ATPase activity. As shown in Figure 10, when ouabain was added to HK-2 cells, dramatic cytoprotection resulted. Of note, neither mitochondrial respiratory chain inhibition nor ouabain seemingly suppressed endocytic transport, as assessed by the Lucifer yellow technique. Hence, decreased myoglobin uptake appears to be an unlikely explanation for the protection observed with these agents. Thus, the available data strongly suggest that cellular respiration, in general, and terminal mitochondrial electron transport, in particular, are key determinants of myoglobin's cytotoxic effect. The reason for this cannot be stated. However, it appears likely that decreased formation of putative cytotoxic H_2O_2/Fe based reactive intermediates [52] at the site of terminal electron transfer to molecular oxygen is responsible. It is noteworthy that Brezis et al have done much to advance the theory that cellular metabolic work (Na transport) is a critical determinant of toxic and hypoxic medullary thick ascending limb tubular cell death [56]. They have related much of its impact to ATP consumption and hence, energy availability. The present study indicates that: (a) transport activity can also alter the expression of proximal, and not just distal, tubule injury; and (b) reductions in oxidant stress and not just energy availability may be involved.

Obviously, cell culture studies have their limitations, making extrapolation of the present data to previously obtained *in vivo* results a subject of potential controversy. Furthermore, since conclusions drawn from the present study were based on results obtained using chemical inhibitors/stimulators of biologic processes, and since such probes could exert nonspecific effects, potentially misleading data could have been obtained. Indeed, the latter consideration is why a major effort was made in this study to use different types of inhibitors and in multiple doses wherever possible. Finally, it remains possible that results obtained with HK-2 cells might not be directly applicable to data obtained by others using other models of heme protein toxicity. Nevertheless, that many striking similarities exist between our current results and those that we have obtained previously with whole animal and isolated tubule experiments (for example, DFO, Sn protoporphyrin mediated protection [19]; decreased oxidant injury with site 2/3 respiratory chain inhibitors [20]; worsened oxidant stress with GSH [19]; and negative results with ·OH scavengers [14, 16]) implies that the present results may, in fact, have relevance.

In conclusion, the present study provides the following potential new insights into the subcellular determinants of myoglobin

cytotoxicity, as assessed with a cultured human proximal tubular cell system. (1) HO is a critical determinant of myoglobin-induced injury, as evidenced by the fact that HO inhibition blocks myoglobin's cytotoxic effect. (2) HO presumably mediates this injury via free iron release, since iron chelation therapy and HO inhibition induced comparable degrees of cytoprotection. (3) myoglobin, rather than cytochrome p450, appears to be a more likely source of the toxic iron, since agents that reportedly stabilize labile cytochrome p450 iron exerted no protective effect. (4) Catalase confers almost total protection against myoglobin cytotoxicity, implicating H_2O_2 as a critical determinant of myoglobin-initiated cell death. That ·OH scavengers were not protective suggests that non-·OH, H_2O_2 catalyzed, reactive iron intermediates are involved. (5) Once released, free iron (as well as intracellular Ca^{2+}), can mechanistically contribute to H_2O_2 generation. Since H_2O_2 liberates iron from porphyrin rings, this can serve as a positive feedback loop, perpetuating iron-mediated toxicity. (6) Terminal mitochondrial electron transport appears to be a critical site of cytotoxic free radical generation during myoglobin-induced HK-2 cell attack. Presumably, iron-based/ H_2O_2-driven reactive intermediate formation at that site is involved.

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