Intracellular Myoglobin Loading Worsens H₂O₂-Induced, but not Hypoxia/Reoxygenation–Induced, In Vitro Proximal Tubular Injury

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Intracellular iron reportedly mediates many forms of tissue injury, including ischemic and myohemoglobinuric acute renal failure. This action may be explained by the ability of iron to catalyze the formation of the highly toxic hydroxyl radical (·OH) from H₂O₂ via the Fenton/Haber-Weiss reactions. To assess whether renal tubular myoglobin/iron loading, induced by a physiological mechanism (endocytosis), alters its susceptibility to O₂ deprivation/reoxygenation– and H₂O₂-mediated injury, rats were infused with myoglobin or its vehicle (5% dextrose, control rats), and after 2 hours, proximal tubular segments (PTSs) were isolated for study. This infusion caused substantial myoglobin endocytic uptake (~25 µg/mg PTS protein), and it doubled PTS catalytic iron content (assessed by bleomycin assay). Nevertheless, PTS viability (percent lactate dehydrogenase release) was minimally affected (4% to 6% increase), and an increased ·OH burden (assessed by the salicylate trap method) did not appear to result. Deferoxamine addition, reported to protect against in vivo acute renal failure, paradoxically increased ·OH levels (~25%) in myoglobin-loaded, but not control, PTSs. Conversely, dimethylthiourea (an ·OH scavenger) depressed ·OH (by ~80%) in all PTSs. Myoglobin/iron loading modestly increased PTS vulnerability to exogenous H₂O₂ addition (P < .001). However, tubular susceptibility to hypoxia (15 and 30 minutes)/reoxygenation injury was not affected. ·OH levels appeared to fall in response to both forms of injury, suggesting decreased ·OH production and/or ·OH scavenging. To assess whether myoglobin decreases ·OH levels in the presence of Fenton reactants, myoglobin and six other test proteins were incubated with Fe⁺/H₂O₂. Myoglobin decreased ·OH levels by ~70%, a significantly greater decrement than was observed with the other proteins tested. Conclusions are as follows: (1) Myoglobin loading increases PTS catalytic iron content, predisposing to H₂O₂-mediated injury, (2) Myoglobin and deferoxamine can exert antioxidant and pro-oxidant effects, respectively. (3) PTS myoglobin loading does not worsen hypoxia/reoxygenation injury, suggesting that myoglobinuria does not exacerbate in vivo ischemic acute renal failure by a direct proximal tubular cell effect. (Circ Res. 1993;73:926-934.)

Key Words • hydroxyl radical • antioxidants • deferoxamine • salicylate • acute renal failure

In recent years, it has been widely proposed that oxygen free radical formation with resultant oxidant tissue stress is a critical mediator of ischemia/reperfusion injury of multiple organs, including the kidney (eg, see References 1 and 2). It has also been suggested that tissue iron, if released from intracellular proteins, can dramatically accentuate this pathway of injury both by directly participating in free radical formation (ferryl/perferryl ion) and by catalyzing the conversion of H₂O₂ to the highly toxic hydroxyl radical (·OH) via the Fenton/Haber-Weiss reactions. Since both renal ischemia and renal iron overload may occur during myohemoglobinuric acute renal failure (ARF), these injury pathways may be especially important in the evolution of this disease. In support of this view are the following: (1) Iron chelation (deferoxamine [DFO]) and ·OH scavenger (dimethylthiourea [DMTU] benzoate) therapy may mitigate myohemoglobinuric, as well as ischemic, ARF. (2) Both myohemoglobinuric and ischemic ARF reportedly cause lipid peroxidation, a consequence of oxidant tissue stress. (3) Myohemoglobinuric ARF is associated with increased renal production of H₂O₂, which can liberate iron from heme proteins. (4) Infusion of either intravenous iron (FeCl₂-EDTA) or heme proteins can exacerbate experimental ischemic ARF.

Despite these considerations, problems inherent in experiments in vivo (such as those above) make it difficult to conclude that proximal tubular cell iron, either in normal or excess concentrations, participates in either heme protein or ischemic ARF by directly altering tubular integrity. For example, FeCl₂-EDTA, heme protein, and antioxidant effects on the kidney could be mediated primarily by hemodynamic or intraluminal influences rather than by directly influencing proximal tubular cell events. In addition, there is no experimental proof that intravenous Fe-EDTA or heme protein infusion actually increases intracellular proximal tubular free iron content. Finally, even if intracellular free iron loading were to occur under these conditions, whether the iron exists in a molecular form

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or at a subcellular location, which permits it to catalyze critical free radical reactions, is open to question.

Given these uncertainties as to the influence of iron on experimental ischemic and myohemoglobinuric ARF, this laboratory recently assessed the direct effect of catalytic (non–protein bound) iron on in vitro proximal tubular cell responsiveness to O₂ deprivation/reoxygenation injury. To this end, isolated rat proximal tubular segments (PTSs) were subjected to hypoxia/reoxygenation injury, either in the presence or absence of 2 mmol/L FeSO₄ or FeCl₃ added directly to the PTS suspension. Although both iron salts caused marked PTS lipid peroxidation, each paradoxically mitigated hypoxic cell killing. Based on these findings, the generally held view that catalytic iron directly potentiates O₂ deprivation/reoxygenation injury seems open to question.

Two potentially important facts need to be considered when interpreting the experiments described above. First, the iron concentration used (2 mmol/L) far exceeded a physiologically relevant range (nanomolar to micromolar). Thus, it is possible that iron could have exerted a number of non-specific pharmacological influences that overrode adverse free radical effects. Second, despite the massive extracellular iron additions, there was no proof that the iron reached critical intracellular locations. Of note, -OH is highly reactive, causing injury at the site of its production. Thus, the failure of exogenous iron to worsen in vitro hypoxia/reoxygenation injury could have been due to its inability to reach physiologically relevant intracellular targets.

Given these uncertainties and in light of the widespread interest in the role of iron in cell injury, in general, and in ischemic and myohemoglobinuric ARF, in particular, the present study was undertaken to address the following specific questions: (1) Does myohemoglobinuria actually produce proximal tubular cell free iron loading, and if so, to what degree? (2) If proximal tubular free iron loading does result, is it in a form that permits it to catalyze critical free radical–based (eg, the Fenton/Haber-Weiss) reactions? (3) If myoglobin–liberated iron is, in fact, “catalytic,” does its presence cause a potentiation of O₂ deprivation/reoxygenation injury, as previously suggested? Since DFO and -OH scavengers are reported to mitigate in vivo myohemoglobinuric ARF, do these agents actually decrease -OH concentrations within myoglobin–laden proximal tubular cells, supporting the concept that this is the reason for their protective effects? To address these issues, rat proximal tubules were myoglobin–loaded in vivo. After the tubules were isolated for in vitro study, -OH levels and cell viability were assessed in response to oxygenation and hypoxia/reoxygenation and during exogenous H₂O₂ addition. The results of these experiments form the basis of this report.

Materials and Methods

**In Vivo Myoglobin Loading and PTS Isolation**

Male Sprague Dawley rats (weight, 175 to 300 g; Charles River Laboratories, Wilmington, Mass) were anesthetized with pentobarbital (30 to 40 mg/kg IP), and a PE-50 jugular venous catheter was inserted. Myoglobin loading of renal tubular cells was induced by infusing 50 mg purified horse skeletal muscle myoglobin (M-0630, Sigma Chemical Co, St Louis, Mo) per 100 g of body weight at a constant rate over 2 hours (30 mg myoglobin per milliliter of 5% dextrose/water). Infusion of this myoglobin preparation (≈80% ferrymyoglobin; iron content, 0.3%; >97% pure) in this, or lesser, amounts has previously been shown by this laboratory to markedly potentiate superimposed ischemic tubular necrosis and the severity of filtration failure without altering blood pressure or renal blood flow. On the basis of these findings, this preparation was selected for use in this investigation. Controls for these myoglobin–treated rats consisted of rats infused with an equal volume of 5% dextrose/water (the myoglobin vehicle) over a 2-hour period. At the end of the infusions, the kidneys were resected through a midline abdominal incision, and they were immediately placed in ice-cold buffer (buffer A consisting of [mmol/L] NaCl, 115; KCl, 2.1; NaHCO₃, 25; KH₂PO₄, 2.4; CaCl₂, 1.2; MgSO₄, 1.2; and MgCl₂, 1.2; pH 7.4; previously gassed with 95% O₂–5% CO₂). Then PTSs were isolated, essentially as previously described (Reference 16; no DFO was used in the isolation process). In brief, the renal cortices were dissected on a cooled surface, minced, and placed in 5 mL of buffer identical to that above except for 2.5 mmol/L CaCl₂ and 1.2 mmol/L phosphate concentrations plus the addition of 25 mmol/L mannitol, 2.5 mg/mL fatty acid–free bovine serum albumin, 5 mmol/L glucose, 4 mmol/L sodium lactate, 1 mmol/L alanine, 10 mmol/L sodium butyrate, and 1.5 mg/mL collagenase D (Boehringer-Manheim, Indianapolis, Ind). The final buffer pH, before collagenase addition, was adjusted to 6.5 by 1N HCl addition. The tissues were digested at 36°C in a rotating water bath for approximately 30 minutes, the flask was opened, 30 mL of ice cold buffer A was added, and then the digested tissue was passed through a stainless-steel sieve and centrifuged. The pellet was washed in 30 mL of fresh buffer A and then in 10 mL of buffer A containing 5% bovine serum albumin. The final pellet was added to 31% Percoll (buffer A) and centrifuged (2000g for 10 minutes, 4°C), with viable PTSs being recovered at the bottom of the tube. This material was washed twice in ice-cold buffer A to remove the Percoll, and the final pellet was suspended in an incubation medium consisting of (mmol/L) NaCl, 100; KCl, 2.1; NaHCO₃, 25; KH₂PO₄, 2.4; CaCl₂, 1.2; MgCl₂, 1.2; MgSO₄, 1.2; glucose, 5; sodium butyrate, 10; alanine, 1; and sodium lactate, 4; along with 0.6% dextran T₅₀₀ (gassed with 95% O₂–5% CO₂; final pH 7.40). The PTS suspension (2 to 5 mg protein/mL) was warmed from 4°C (isolation temperature) to 36°C (experimentation temperature) for 15 minutes in a shaking water bath, and then a baseline percent lactate dehydrogenase (LDH) release was determined as an index of PTS viability (eg, see References 15 and 16). LDH determinations were performed using autoanalyzer technology (Cobas BIO, Roche Laboratories, Branchburg, NJ).

**Effect of Myoglobin Loading on PTS Response to O₂ Deprivation/Reoxygenation Injury**

Fourteen PTS preparations, seven from myoglobin–infused rats and seven from control rats, were each divided into four 2.5–mL aliquots and placed into 25-mL siliconized Erlenmeyer flasks and treated as follows: (1) continuous oxygenation (95% O₂–5% CO₂ incubation at 36°C for 45 minutes), percent LDH release being
determined after 15, 30, and 45 minutes (determined on 150-μL aliquots); (2) mild hypoxia/reoxygenation injury (15 minutes of hypoxia with 95% N2–5% CO2, followed by 30 minutes of reoxygenation), percent LDH release being determined at the end of each period; (3) severe hypoxia/reoxygenation injury (30-minute hypoxia/15-minute reoxygenation), percent LDH release being determined at the end of each period; and (4) one PTS aliquot from each of these preparations, used as described below.

Effect of Myoglobin Loading on H2O2-Induced PTS Cytotoxicity

Since H2O2-mediated PTS toxicity is iron dependent (eg, see References 17 through 19), the response of myoglobin-loaded and control PTSs to exogenous H2O2 addition was tested. To this end, the fourth aliquot from each of the above-described PTS preparations was incubated under oxygenated conditions in the presence of 15 mmol/L H2O2 (an amount necessary to induce any degree of cell death). Percent LDH release was assessed at the end (45 minutes) of each experiment.

Assessment of PTS Catalytic (Free) Iron and Myoglobin Content

To ascertain whether myoglobin infusion results in increased PTS-free iron content, the continuously oxygenated PTS aliquot from each of the above PTS preparations was assayed for catalytic iron by the bleomycin assay of Gutteridge and Hou,26 as previously described.21 In brief, this assay is based on the principles that (1) bleomycin complexes non-protein-bound iron, (2) this complex can be quantified by its ability to induce oxidant DNA damage, and (3) this damage directly correlates with the initial free iron concentration. Corresponding PTS myoglobin concentrations were assessed by Drabkin's test (Sigma), using myoglobin as the standard.

Assessment of PTS -OH Levels in the Presence and Absence of Myoglobin Loading

The following experiments were undertaken to ascertain whether (1) myoglobin-loaded PTSs generate excess -OH, (2) DFO alters its production, and (3) DMTU, reported to protect against ischemic and myoglobinemic ARF,29 functions as an effective intracellular -OH scavenger. Five separate myoglobin-loaded and five control PTS preparations were each divided into three aliquots: (1) continuous oxygenation for 75 minutes, (2) continuous oxygenation in the presence of 2 mmol/L DFO, and (3) continuous oxygenation in the presence of 10 mmol/L DMTU. Sodium salicylate, a stable -OH trap, was added to each aliquot (2 mmol/L) at the start of the incubations. After 75 minutes, percent LDH release was determined, and then sample aliquots were lysed and analyzed for hydroxylated salicylate byproducts (2,3- and 2,5-dihydroxybenzoic acid [2,3- and 2,5-DHBA, respectively]) by high-performance liquid chromatography using electrochemical detection.21,22 As previously reported by this laboratory and others, the degree of 2,3- and 2,5-DHBA production is an index of -OH generation and concentrations.17,21-28

The following experiment assessed whether myoglobin-loaded PTSs generate excess -OH either during hypoxia/reoxygenation and/or after exogenous H2O2 addition. Five PTS preparations, made from myoglobin-infused rats, were each divided into three aliquots: (1) continuous oxygenation for 45 minutes, (2) hypoxia (22.5 minutes)/reoxygenation (22.5 minutes), and (3) continuous oxygenation for 45 minutes with 15 mmol/L H2O2 addition (twice) at the start and at the midpoint of the 45-minute incubation. Incubations occurred in the presence of 2 mmol/L sodium salicylate. Percent LDH release and DHBA concentrations were assessed after 45 minutes.

Evaluation of the Effect of Myoglobin on -OH Levels in the Presence of Fenton Reactants

Because the above experiments suggested that myoglobin loading paradoxically decreases PTS -OH burdens, the following study analyzed whether myoglobin can decrease -OH levels in the presence of Fenton reactants. To achieve this goal, -OH-generating reactants (1 mmol/L FeSO4 + 15 mmol/L H2O2 + 10 mmol/L ascorbate) were added to the PTS buffer (with 2 mmol/L salicylate, no PTSs) and incubated (36°C) either in the presence or absence of one of the following proteins (25 mg/mL): myoglobin, bovine serum albumin (A-6003), gamma globulin (A-2387), ribonuclease (R-5503), cytochrome C (C-2506), hemocyanin (H1757) (from Sigma), or egg white lysozyme (Boehringer-Mannheim). DHBA concentrations were assessed after completing 30-minute incubations (n=4 to 6 per test sample).

Calculations, Statistics, and Renal Histology

All values are given as mean±SEM. Statistical comparisons were made by paired or unpaired Student’s t test unless otherwise stated. If multiple comparisons were made, the Bonferroni correction was applied. Since DHBA production by PTSs is linearly related to the amount of PTSs present (as assessed by total suspension LDH concentrations), the DHBA values are reported as nanomoles per 100 LDH units. For the sake of reference, 100 LDH units in this laboratory equate with 1500 LDH units, using a reference enzyme (No. 340-10, Sigma) and is equivalent to ~1.8 mg PTS protein per milliliter suspension, as determined by the bicinchoninic acid method (Pierce Chemical Co, Rockford, Ill). The validity of factoring DHBA concentrations by LDH units is based on a near-perfect linear relation between DHBA production under oxygenated conditions and total LDH concentrations (r=.97).

To make certain that myoglobin infusion had induced tubular cell lysosomal myoglobin uptake, renal cortex from a typical myoglobin-infused rat was examined by light and transmission electron microscopy.

Results

Effects of Myoglobin Infusion on Renal Myoglobin and Catalytic Iron Accumulation

After completing 2 hours of myoglobin infusion, the kidneys were deeply pigmented with additional areas of punctate myoglobin accumulation observable on the cortical surfaces (Fig 1). Light microscopy of a typical kidney revealed frequent distal tubular myoglobin cast formation and no apparent proximal tubular damage. Electron microscopy demonstrated extensive and generalized proximal tubular cell protein incorporation within the lysosomal system (Fig 2), confirming that
intracellular myoglobin loading had occurred. In other respects, the tubular cells appeared normal. After completing the PTS isolation process, the pelleted PTS preparations obtained from myoglobin-infused rats were visibly stained with heme pigment compared with control PTS preparations. Quantification of their heme protein content with Drabkin’s reagent indicated a concentration of 49±4 μg per milligram PTS protein. In contrast, the “heme protein” content of control PTSs was 24±4 μg per milligram PTS protein (P<.01 versus myoglobin-loaded PTSs), yielding a difference of 25 μg per milligram total PTS protein (equating to ~1.5 nmol myoglobin per milligram PTS protein). Bleomycin-detectable free iron concentrations were significantly higher for the myoglobin-treated versus the control PTSs, both in terms of absolute lysed PTS suspension iron concentrations (31±3 versus 13±2 μmol/L, P<.005) and after expressing these values per 100 LDH units (myoglobin PTSs, 14.9±1.4; control PTSs, 7.5±0.6; P<.001) and as nanomoles iron per milligram PTS total protein (7.6±0.5 versus 4.1±0.2, P<.001).

Effect of Myoglobin Loading on PTS Viability During Oxygenation and With Hypoxia/Reoxygenation Injury

PTS recovery and LDH release during continuous oxygenation. Myoglobin infusion did not significantly alter PTS recovery from kidneys, as reflected by comparable total LDH concentrations for the myoglobin-treated and the control groups (total LDH concentrations in final suspension: myoglobin-treated group, 216±21 U/mL; control group, 182±20 U/mL; P=NS). Baseline PTS viability, as assessed by percent LDH release after completion of the 15-minute rewarming period, was essentially identical for the two PTS groups (see Fig 3). After completing 15, 30, and 45 minutes of oxygenation, there was a trend toward minimally higher LDH release...
from the myoglobin-loaded versus the control PTSs. However, this only reached statistical significance at the 45-minute time point (myoglobin-loaded PTSs, 18±2%; control PTSs, 14±1%; P<.025).

Mild hypoxic injury. After 15 minutes of hypoxia, percent LDH release for myoglobin-treated and control PTSs was 18±2% and 16±2%, respectively (P=NS). Percent LDH release for these two groups still did not statistically differ after completing 30 minutes of reoxygenation (myoglobin-treated group, 28±3%; control group, 24±2%). Fig 4A depicts the degree to which 15 minutes of hypoxia and 30 minutes of reoxygenation increased the percent LDH release over the values observed in the time-matched oxygenated aliquots (corrected LDH release, eg, percent LDH released after 15 minutes of hypoxia minus percent LDH released after 15 minutes of oxygenation). When so analyzed, identical degrees of hypoxia/reoxygenation injury were observed for the myoglobin-treated and control groups.

Severe hypoxic injury. Thirty minutes of hypoxia induced 46±4% and 51±3% LDH release from the control and myoglobin-loaded PTSs, respectively (P=NS). After 15 minutes of reoxygenation, these values increased to 54±3% and 58±2% LDH release, respectively (P=NS). As depicted in Fig 4B, the corrected percent LDH release (ie, the rise over the values observed in the time-matched oxygenated control group) indicated that the control and myoglobin-loaded PTSs experienced identical degrees of hypoxia/reoxygenation–induced LDH release by the end of the experiments (each 40±2%).

PTS Vulnerability to 15 mmol/L H2O2 Addition

After completing 45 minutes of oxygenated incubation in the presence of H2O2, the myoglobin-loaded PTSs demonstrated significantly greater LDH release than did the control PTSs (P<.001, see Fig 5). This enhanced vulnerability to H2O2 was also apparent when the data were expressed as increments over the values observed in the coincubated oxygenated control group (ie, corrected LDH values).

Salicylate Trap Assessment of -OH Levels in Oxygenated PTSs

PTS incubated in the absence of antioxidants. The 2.5- and 2.3-DHBA concentrations in continuously oxygenated PTSs are presented in Fig 6. In the absence of DFO or DMTU, no excess production/availability of -OH was apparent in the myoglobin-loaded PTSs since both the 2.5- and 2.3-DHBA concentrations were slightly lower, rather than higher, in the myoglobin-treated versus the control group.

PTS incubated with DFO. DFO addition to control PTSs did not significantly alter either 2.5- or 2.3-DHBA production (total DHBA [2.5-+2.3-DHBA]: with DFO, 1.59±0.16 nmol/100 LDH units; without DFO, 1.52±0.16 nmol/LDH units; P=NS; see Fig 6). However, DFO addition to myoglobin-loaded PTSs paradox-
ically increased both 2,5- and 2,3-DHBA concentrations (by 25% and 32%, respectively, over values observed in the coincubated myoglobin-loaded/none-DFO-exposed PTs; P<.02). Total DHBA concentrations for these myoglobin-loaded PTs with and without DFO addition were 1.43±0.10 and 1.17±0.11 nmol/100 LDH units, respectively (P<.02). However, even with this DFO-driven DHBA increment, the DHBA levels still did not exceed those observed in the normal PTs.

PTTs incubated with DMTU. Addition of DMTU, an -OH scavenger, caused marked suppression of both 2,5- and 2,3-DHBA production by both the control and myoglobin-loaded PTs (Fig 6). The degree of suppression was greater for 2,5-DHBA than for 2,3-DHBA (85±1% versus 59±5%, respectively; P<.001), confirming that in PTs 2,5-DHBA is at least comparable to 2,3-DHBA as a marker of -OH-driven oxidant stress. 17

Overall statistical analysis of DHBA production in myoglobin-loaded versus control PTs during oxygenation. As depicted in Fig 6, comparisons of 2,5- and 2,3-DHBA levels for the myoglobin-loaded versus the control PTs revealed slightly lower concentrations in the myoglobin-loaded group in every instance, although no individual comparison yielded a statistical difference. However, an overall analysis of all of the treatment groups (myoglobin-loaded versus control PTs) indicated significantly less DHBA generation with myoglobin treatment (P<.015 by signed rank test).

LDH release with and without DFO/DMTU addition. Neither DFO nor DMTU significantly altered LDH release in the above experiments. For the control PTs, the following results were obtained after 75 minutes of incubation: no addition, 15±2%; DFO, 17±3%; and DMTU, 17±2%. The corresponding values obtained for the myoglobin-loaded PTs were as follows: 21±4%, 17±2%, and 20±3%, respectively.

Salicylate Trap Assessment of -OH During Hypoxia/Oxygenation and H2O2 Addition

Both hypoxia/reoxygenation and H2O2 addition induced significant LDH release (27±4% and 29±3%, respectively) compared with the continuously oxygenated control group (14±2%, P<.015). Despite this evoking cell injury, both hypoxia/reoxygenation and H2O2 significantly decreased, rather than increased, both 2,5- and 2,3-DHBA generation (the final levels of which reflect a summation value for the entire experiment). Total DHBA values for these groups by the end of the experiments were as follows: control group, 1.01±0.12 nmol/100 LDH units; hypoxia/reoxygenation group, 0.61±0.08 nmol/100 LDH units; and H2O2 group, 0.59±0.08 nmol/100 LDH units; the latter two being significantly lower than the control group (P<.02) (see Fig 7). These reductions in DHBA (≈40%) were, on a percent basis, approximately three times greater than the increments in percent LDH release (≈14%), indicating that a loss of viable cells, per se, could not explain the reduced DHBA production.

Myoglobin/Protein Effects on Fe2+/H2O2-Mediated DHBA Production

Fe2+/H2O2/ascorbate, in the absence of protein addition, caused marked 2,5- and 2,3-DHBA generation (from essentially zero DHBA to a total of 23±2 nmol/mL, 60%/40%, 2,5-/2,3-DHBA, respectively). Myoglobin suppressed this DHBA production (by ≈70%), far more than any other test protein (Fig 8). Hemoglobin-stimulated DHBA production (from 23±2 to 44±4 nmol/mL) despite the fact that in the absence of Fe2+/H2O2/ascorbate it caused virtually no DHBA generation (≈1 nmol/mL).

Discussion

Recently, there has been substantial interest in the role of heme protein–derived intracellular free iron as a
promoter of oxidant cell stress, in general, and as a mediator of ischemic/reperfusion injury, in particular. Past in vivo and in vitro studies designed to address these issues have been hindered because in vivo experiments rarely provide information that can be directly extrapolated to primary cellular events and because in vitro studies of iron toxicity, which manipulate iron concentrations by exogenous iron addition or iron chelator therapy, are open to question since these interventions may fail to achieve the desired results at critical intracellular locations. Thus, the role of iron in the evolution of ischemic and heme protein–induced tubular necrosis has remained ill defined. Because of this, the present study was undertaken to directly assess whether heme protein loading of proximal tubules increases their catalytic iron content, whether this change predisposes to hypoxia/reoxygenation– and H$_2$O$_2$–induced cell injury, and if so, whether an increased -OH burden is responsible.

Because exogenous myoglobin or free iron addition to PTS suspensions does not ensure that intracellular targets are exposed, rats in the present study were subjected to intravenous myoglobin infusion for 2 hours, and then their proximal tubules were isolated for investigation. As shown in Fig 1, myoglobin infusion caused marked, and generalized, renal cortical myoglobin accumulation, and electron microscopy (Fig 2) confirmed that proximal tubular myoglobin incorporation within the endocytic-lysosomal system had occurred. Additional evidence for substantial PTS myoglobin loading included the following: a heme protein increment of 25 μg/mg PTS total protein; PTS, after exhaustive washings, visibly stained with heme pigment; and bleomycin analysis indicating an approximate doubling of “catalytic” free iron content. This latter observation is particularly important since it documents, for the first time, that a consequence of proximal tubular heme protein loading is increased intracellular catalytic iron activity. It is noteworthy that the free iron increment (3.5 nmol per milligram protein) approximated, or was greater than, the amount of myoglobin uptake (=1.5 nmol per milligram PTS protein). This suggests that (1) catalytic iron is very rapidly liberated from myoglobin, once achieving an intracellular location; (2) proximal tubular heme protein overload causes sublethal cell injury, which secondarily releases iron from normal intracellular iron binding proteins; or (3) circulating and/or intraluminal catalytic iron, which has previously been demonstrated in myohemoglobinuric states, is extracted by proximal tubules, thereby causing, or contributing to, the cell catalytic iron increment.

To ascertain whether myoglobin, and hence catalytic iron loading, does, in fact, directly impact on proximal tubular cell susceptibility to O$_2$ deprivation/reoxygenation injury, PTSs, harvested from control and myoglobin-infused rats, were subjected to either mild (15-minute) or moderately severe (30-minute) hypoxic insults, and cell injury was assessed (LDH release) on completion of the hypoxic and reoxygenation periods. As depicted in Fig 4, the myoglobin-loaded and control PTSs sustained essentially identical degrees of cell injury, irrespective of the time of LDH assay or the protocol used. Of note, this laboratory has previously demonstrated that PTS iron depletions (iron chelators) does not protect against hypoxia/reoxygenation injury and that 2 mmol/L iron addition to PTS buffer, if anything, exerts a modest cytoprotective effect. Thus, the present results, obtained using a highly relevant technique for inducing cell iron loading, further support the view that catalytic iron is not a critical mediator of proximal tubule O$_2$ deprivation/reoxygenation injury. In addition, the fact that myoglobin loading did not worsen PTS hypoxia/reoxygenation injury implies that myoglobinemia/uria, well known to worsen in vivo ischemic ARF (e.g. see References 6, 8, 13, and 14), may do so by inducing adverse intraluminal/intravascular influences rather than by directly altering proximal tubular cell events.

During the design of the present experiments, there was a concern that in vivo myoglobin loading would lead to a “selection artifact,” i.e., that a large number of myoglobin-loaded cells would die during PTS preparation, leaving behind a highly resistant cell population for study. If true, this result potentially could have masked myoglobin/iron effects on hypoxia/reoxygenation injury. Alternatively, in vivo myoglobin loading theoretically could have produced a severely compromised PTS population, thereby causing an overestimate of their susceptibility to superimposed hypoxia/reoxygenation damage. However, neither concern appeared substantiated for the following reasons: (1) The PTS yield was comparable in the myoglobin-infused and control rats (based on LDH totals), indicating no selection artifact. (2) Baseline PTS viability, assessed by LDH release after rewarming, was essentially identical for the myoglobin-treated and control groups. (3) After 45 minutes of oxygenated incubation, the myoglobin-loaded PTSs demonstrated only 4% greater LDH release than the control PTSs. Thus, it appears that sufficiently stable and representative PTS preparations were obtained to permit the above conclusions to be drawn.

Because in vivo myohemoglobinuric ARF is reportedly associated with increased renal H$_2$O$_2$ production and because myoglobin infusion enhanced the PTS catalytic iron content, the next goal of the present study was to assess whether this increased iron burden predisposed PTSs to H$_2$O$_2$-mediated injury. Such a result would be predicted, since it has been well established...
that H2O2 cytotoxicity is free iron dependent (see References 17 through 19). Thus, control and myoglobin-loaded PTSs were subjected to exogenous H2O2 addition, and as demonstrated in Fig 5, a modest increment in cell injury was noted in the myoglobin-loaded group. Of note, Shah and colleagues7,8,10,30 have previously suggested that both iron and H2O2 are important mediators of myohemoglobinuric ARF. The present in vitro data support this conclusion and strongly suggest that an adverse iron-H2O2 interaction can occur directly at the proximal tubular cell level.

A presumptive explanation for why myoglobin loading increases PTS susceptibility to H2O2 addition, and to in vivo ischemic ARF, is that catalytic iron can generate highly toxic -OH from H2O2 via the Fenton/Haber-Weiss reactions. Previous research from this laboratory, using normal or iron-depleted PTSs, has suggested that H2O2 cytotoxicity and hypoxia/reoxygenation injury are not necessarily -OH dependent.17,21,29 However, myoglobin loading conceivably could alter these injury pathways by providing excess iron for -OH generation. To test this hypothesis, control and myoglobin-loaded PTSs were incubated under a variety of experimental conditions in the presence of sodium salicylate. Since the latter is actively transported by proximal tubules and serves as a stable -OH trap, hydroxylated salicylate byproduct (2,5- and 2,3-DHBA) generation reflects the tubular cell -OH burden,21 as indicated by the present observation that DMTU, an -OH scavenger, markedly depressed both 2,5- and 2,3-DHBA generation. Application of the salicylate trap technique to the present study yielded a number of noteworthy results. First, myoglobin loading tended to depress, rather than enhance, PTS DHBA production under oxygenated conditions, suggesting either decreased -OH generation or reduced -OH bioavailability, as discussed below. However, irrespective of its cause, the lack of excess DHBA generation under oxygenated conditions indicates that myoglobin/catalytic iron loading of tubules need not necessarily translate into increased -OH-driven oxidant stress. Second, despite the fact that H2O2 addition caused increased cytotoxicity in the myoglobin-loaded PTSs, this enhanced injury correlated with falling, not rising, DHBA concentrations. Previous research from this laboratory has shown that H2O2 cytotoxicity, when studied using normal PTSs, occurs without any significant change in DHBA generation.17 Thus, the present finding of marked reductions in DHBA production during H2O2 addition indicates that myoglobin loading can paradoxically decrease -OH concentrations. Third, despite myoglobin/iron loading, hypoxia/reoxygenation also depressed DHBA production compared with the continuously oxygenated counterparts. Of note, hypoxia/reoxygenation injury of normal PTSs is also associated with decreased -OH generation, undoubtedly because O2 deprivation temporarily limits oxygen free radical formation.21 That this result was reproduced even in myoglobin/iron-loaded PTSs further indicates that PTS hypoxia/reoxygenation injury is not -OH dependent. Fourth, DFO addition to myoglobin-loaded, but not to control, PTSs significantly increased (=25%) DHBA generation. This laboratory previously reported that DFO, when added to extracellular 5 mmol/L FeSO4, augments DHBA production presumably because it accelerates Fe2+ oxidation to Fe3+, the chelated form.17 However, whether DFO could exert this "pro-oxidant" effect at an intracellular location was not defined. The present results suggest that this is indeed the case since DFO addition significantly increased both 2,5- and 2,3-DHBA production in myoglobin-loaded, but not in control, PTSs. This raises the very real possibility that the cytoprotective influence of DFO on in vivo models of ARF may be exerted despite its potential pro-oxidant effect.

The fact that H2O2 addition to myoglobin-loaded PTSs markedly decreased DHBA generation raises two intriguing issues: (1) What, then, is the mechanism by which myoglobin loading predisposes to H2O2 toxicity? (2) What is the explanation for the apparent decrease in -OH concentrations? In regard to the first issue, it is quite possible that H2O2, which characteristically releases iron from intracellular binding proteins,14 causes marked iron liberation within myoglobin-laden PTSs. Since H2O2 toxicity is catalytic iron dependent,17-19 an excess iron burden might explain an increase in cell death. In regard to the second issue, the possibility was considered that myoglobin might suppress -OH levels in the presence of iron/H2O2. Thus, the effect of myoglobin addition and DHBA production, induced by FeSO4/H2O2, was assessed. As depicted in Fig 8, myoglobin markedly suppressed DHBA levels, significantly more than any of the other proteins tested. At least two possible explanations for this finding exist. First, myoglobin could have exerted a peroxidase effect,4 decreasing H2O2 concentrations; and second, Mgb could have functioned as an effective -OH scavenger, as previously suggested by experiments from this laboratory that used a different -OH generating system (Fe2+/Fe3+/ADP).15 Irrespective of the exact mechanism, this experiment makes one point clear: myoglobin need not necessarily increase, and can actually decrease, -OH concentrations, supporting the current findings from the myoglobin-loaded PTS experiments.

In summary, the present study provides the following new insights: (1) Myoglobin loading of proximal tubules via endocytosis causes a rapid increase in cell catalytic iron content, the degree of which approximates (on a molar basis) the amount of myoglobin uptake. Nevertheless, spontaneous -OH-driven oxidant stress does not appear to result. (2) Despite this increased catalytic iron/myoglobin burden, proximal tubular susceptibility to hypoxia/reoxygenation injury is not affected. This suggests that excess cellular catalytic iron loading via endocytosis does not directly alter the severity of O2 deprivation/reoxygenation injury and that myoglobinuria probably worsens in vivo ischemic ARF by affecting intraluminal and/or hemodynamic events. (3) Myoglobin-laden PTSs have increased susceptibility to H2O2 toxicity despite falling DHBA generation. This suggests that H2O2 and iron, purported mediators of myohemoglobinuric ARF, can adversely interact directly at the proximal tubular cell level and that -OH is not a likely mediator of this reaction. (4) DFO appears to exaggerate, rather than lessen, -OH generation in myoglobin-loaded PTSs. This suggests that the beneficial influence of DFO on in vivo models of ARF may occur despite its ability to exert a pro-oxidant effect.

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Intracellular myoglobin loading worsens H2O2-induced, but not hypoxia/reoxygenation-induced, in vitro proximal tubular injury.

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