Inorganic Iron Effects on In Vitro Hypoxic Proximal Renal Tubular Cell Injury

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Abstract

Iron-dependent free radical reactions and renal ischemia are believed to be critical mediators of myohemoglobinuric acute renal failure. Thus, this study assessed whether catalytic iron exacerbates O_2 deprivation–induced proximal tubular injury, thereby providing an insight into this form of renal failure. Isolated rat proximal tubular segments (PTS) were subjected to either hypoxia/reoxygenation (H/R: 27:15 min), "chemical anoxia" (antimycin A; 7.5 μM × 45 min), or continuous oxygenated incubation±ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron addition. Cell injury (% lactic dehydrogenase [LDH] release), lipid peroxidation (malondialdehyde, [MDA]), and ATP depletion were assessed. Under oxygenated conditions, Fe^{2+} and Fe^{3+} each raised MDA (~ 7–10×) and decreased ATP (~ 25%). Fe^{2+}, but not Fe^{3+}, caused LDH release (31±2%). During hypoxia, Fe^{2+} and Fe^{3+} worsened ATP depletion; however, each decreased LDH release (~ 31 to ~ 22%; $P < 0.01$). Fe^{2+}-mediated protection was negated during reoxygenation because Fe^{2+} exerted its intrinsic cytotoxic effect (LDH release: Fe^{2+} alone, 31±2%; H/R 36±2%; H/R + Fe^{2+}, 41±2%). However, Fe^{3+}-mediated protection persisted throughout reoxygenation because it induced no direct cytotoxicity (H/R, 39±2%; H/R + Fe^{3+}, 25±2%; $P < 0.002$). Fe^{3+} also decreased antimycin toxicity (41±4 vs. 25±3%; $P < 0.001$) despite inducing marked lipid peroxidation and without affecting ATP. These results indicate that catalytic iron can mitigate, rather than exacerbate, O_2 deprivation/reoxygenation PTS injury. (*J. Clin. Invest.* 1993. 91:702–708.) Key words: adenosine triphosphate • antimycin A • hypoxia • iron • malondialdehyde

Introduction

For almost 50 years it has been recognized that myohemoglobinuria and renal hypoperfusion/ischemia cause synergistic tubular injury, potentially triggering severe acute renal failure (ARF)[1] (1, 2). However, the mechanism(s) by which they do so have remained poorly defined. Three general possibilities have been considered, focusing at the vascular, intraluminal, and tubular cell levels. First, since heme proteins can lower renal blood flow (e.g., references 3 and 4), myohemoglobinuria may exacerbate tubular cell ATP decrements during a superimposed hypotensive/renal vasoconstrictive event. Second, since heme proteins form intraluminal casts under aciduric conditions, a worsening of ischemia-induced intratubular obstruction may result, potentiating filtration failure (5, 6). Third, heme protein endocytosis renders proximal tubular cells highly vulnerable to superimposed ischemic insults (6). Since proximal tubular necrosis is a critical determinant of both heme protein and ischemic ARF, synergistic tubular injury is undoubtedly an important underlying mechanism for the heme protein–ischemic interaction.

Given that heme proteins and ischemia can each induce injury at the vascular, intraluminal, and tubular cell levels, and because secondary interactions can then occur, it has been difficult to dissect out direct mechanisms of synergistic tubular cell injury in whole-animal experiments. For example, pharmacological probes used to study cellular mechanisms of injury (e.g., mannitol, deferoxamine) can yield misleading information since they may exert vascular (7, 8), intraluminal (7–10), and direct tubular cell effects (7, 10–13). An additional complexity stemming from in vivo experiments is that heme proteins, which are not directly cytotoxic (e.g., references 14 and 15), probably need to be "processed" to induce their adverse effects. For example, several in vivo (11–13) and in vitro (10, 15) studies strongly suggest that free iron, released from heme proteins within the circulation (10) and possibly within the kidney, is partially responsible for heme protein cytotoxicity. Thus, a given in vivo intervention might confer cytoprotection by affecting free iron availability (10) without providing direct insights into mechanisms of tubular injury.

In an attempt to circumvent some of these problems, the present study was undertaken to ascertain direct iron effects on O_2 deprivation/reoxygenation injury of isolated rat proximal tubular segments (PTSs). Since neither vascular effects nor cast formation are operative in the PTS system, and because a constant amount of iron can be delivered, it was reasoned that these in vitro experiments might yield new mechanistic insights into the nature of the heme protein/iron–ischemic interaction. To this end, the following specific questions were addressed: Does free Fe^{2+}, by itself cytotoxic (15), render proximal tubular cells highly vulnerable to superimposed O_2 deprivation/reoxygenation injury? Since iron is capable of catalyzing oxygen-based free radical reactions (16, 17), does an exogenous iron challenge drive hypoxic/reoxygenation proximal tubular oxidant stress? Alternatively, does O_2 deprivation limit the ability of iron to promote oxygen-based free radical reactions? and What effect does free iron have on tubular cell energy levels in the presence and absence of superimposed O_2 deprivation? The relevance of this last question stems from in vivo observations that myoglobin infusion lowers renal cortical ATP concentrations, that these ATP decrements are completely blocked by iron chelation (deferoxamine) therapy, and that this iron-me-

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1. *Abbreviations used in this paper:* ARF, acute renal failure; DFO, deferoxamine; LDH, lactic dehydrogenase; MDA, malondialdehyde; PTS, isolated proximal tubular segments.

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diated effect is apparently independent of changes in renal hemodynamics (18). Thus, if free iron directly synergizes O_2 deprivation–mediated tubular ATP depletion, this would provide a potential new insight into the nature of the in vivo heme protein–ischemic interaction. Investigations into each of these three issues form the basis of this report.

Methods

PTS preparation. Normal male Sprague-Dawley rats (150–250 g; Harlan Sprague Dawley, Inc., Indianapolis, IN), maintained under standard vivarium conditions, were used for all PTS preparations, as previously described (10, 15). In brief, the rats were anesthetized with pentobarbital (30–40 mg/kg i.p.), the kidneys were removed without in vivo perfusion, and the cortices were resected and minced with a razor blade at 4°C. The resulting tissues were digested for 30 to 40 min in a collagenase containing buffer to which 1 mM deferoxamine (DFO) had been added to chelate any free iron generated during the mincing/digestion process (thereby preventing iron toxicity from occurring before experimentation) (19). Viable PTS were pelleted through a 31% Percoll gradient, they were washed five times to remove any remaining collagenase and DFO, and then they were suspended to a concentration of 1.5 to 3.0 mg protein/ml in a buffer with the following composition (mM): 100 NaCl, 2.1 KCl, 25 $NaHCO_3$, 2.4 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $MgSO_4$, 5 glucose, 4 Na lactate, 10 Na butyrate, 1 alanine, and dextran T_{40} 0.6% and gassed with 95% O_2/5% CO_2; final pH 7.40. The PTS preparation (using kidneys from either one or two rats, depending on the weight of the animals and the number of aliquots needed for a given experiment) were rewarmed from 4°C (isolation temperature) to 36°C over 15 min. As an assessment of baseline PTS viability, a 150-μl aliquot was used to determine the percent of lactic dehydrogenase (LDH) released (percent of total LDH in the PTS supernatant after centrifugation), after completion of the rewarming process (10, 15).

Effects of cytotoxic Fe^{2+} on PTS vulnerability to hypoxic-reoxygenation injury. Previous studies have indicated that Fe^{2+}, but not Fe^{3+}, is cytotoxic to PTS (15). Thus, to explore the effects of iron toxicity on hypoxia-reoxygenation injury, five separate PTS preparations were each divided into four aliquots and incubated in a 36°C shaking water bath for 42 min as follows: controls: continuous oxygenated (95% O_2/5% CO_2) incubation; hypoxic-reoxygenation incubation (27 min of hypoxia [gassing with 95% N_2/5% CO_2] followed by 15 min of reoxygenation [95% O_2/5% CO_2]); incubation with 2 mM $FeSO_4$ under oxygenated conditions; and hypoxic-reoxygenation injury, as per aliquot 2, in the presence of 2 mM $FeSO_4$. At the completion of the 42-min incubations, cell injury was reassessed by determining the percent of LDH released.

To assess whether Fe^{2+} effects in the above experiments were predominantly expressed during the hypoxic or the reoxygenation period, eight additional PTS preparations were treated exactly as detailed above except that the %LDH release was determined after both 27 and 42 min of each incubation. Hypoxic and reoxygenation injury in aliquots 2 and 4 was gauged by the percent of LDH released during the 0–27- and during the 27–42-min time frames, respectively.

Effect of Fe^{2+} and hypoxic-reoxygenation injury on PTS lipid peroxidation. In four of the above experiments, lipid peroxidation was assessed both after completing the initial 27-min and the final 15-min incubations. To this end, at the time of aliquot sampling for LDH release, an additional 0.25 ml of suspension was removed, and the PTSs recovered by centrifugation in a microfuge, were assayed for malondialdehyde (MDA) by using the thiobarbituric acid method of Uchiyama and Mihara (20), as previously employed in this laboratory (15). Thus, the effects of Fe^{2+}, hypoxia-reoxygenation, and Fe^{2+} + hypoxia-reoxygenation on lipid peroxidation could be assessed. The MDA values were expressed as nmol/mg PTS protein, the latter being determined by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL).

Effect of Fe^{3+} on PTS hypoxic-reoxygenation injury and lipid peroxidation. It has previously been demonstrated that, although Fe^{3+} induces PTS lipid peroxidation, cell killing does not result (15). Thus, the following experiment was performed to ascertain iron effects on hypoxic-reoxygenation injury in the absence of direct iron-mediated cytotoxicity. Five PTS preparations were each divided into four equal aliquots as follows: 42-min oxygenated incubation; 42-min oxygenated incubation in the presence of 2 mM $FeCl_3$ (Fe^{3+}); 27-min hypoxic and then 15 min of reoxygenated incubation; and 27-min hypoxic and then 15-min reoxygenated incubation in the presence of Fe^{3+}. At the completion of the incubations, %LDH release and MDA concentrations were determined as noted above.

Effect of Fe^{2+} and Fe^{3+} on PTS adenine nucleotide concentrations under oxygenated and hypoxic conditions. The following experiment assessed whether iron addition causes PTS ATP losses under oxygenated conditions (as suggested by in vivo experiments; reference 18); whether such losses are sufficient to explain Fe^{2+}-mediated cell killing; and whether iron addition alters hypoxia-induced tubular energy depletion. To these ends, five separate PTS preparations were each divided into six aliquots: continuous oxygenated incubation; oxygenated incubation in the presence of 2 mM Fe^{2+}; oxygenated incubation in the presence of 2 mM Fe^{3+}; hypoxic incubation; hypoxic incubation + 2 mM Fe^{2+}; and hypoxic incubation + 2 mM Fe^{3+}. After completing 27 min under these conditions, an 150-μl sample was removed for assessing LDH release, DFO (Ciba Pharmaceuticals Company, Summit, NJ) was immediately added to each aliquot (final concentration, 4 mM), and then the adenine nucleotides were extracted by adding 66.6% TCA (final concentration, 6.66%). (Note: These three maneuvers [aliquot sampling and DFO and TCA addition] were performed while maintaining continuous hypoxic conditions. DFO was added because in pilot studies it was documented that free iron partially interferes with adenine nucleotide recovery from TCA, a phenomenon that is prevented by DFO addition). The PTS suspensions were vortexed, centrifuged, and the supernatants extracted in freon-trioctylamine to remove TCA (18). The supernatants were analyzed for ATP, ADP, and AMP by HPLC (21), as previously described (18). All values were expressed as nmol/mg PTS protein.

Effect of Fe^{2+} plus DFO on hypoxic-reoxygenation injury. In each of the above experiments, Fe^{2+} or Fe^{3+} induced marked lipid peroxidation. To assess whether lipid peroxidation is a critical determinant of iron effects on hypoxic-reoxygenation injury, the following experiment was performed. Four sets of PTS were subjected to the hypoxia-reoxygenation protocol with either buffer only addition (controls), DFO addition (4 mM), or DFO followed immediately by 2 mM Fe^{2+} addition. Excess DFO relative to the amount of Fe^{2+} was added to limit lipid peroxidation (15), but potentially leaving iron (possibly in the feroxamine complex) available to confer a protective effect. After completing the 42-min incubations, %LDH release and MDA concentrations were determined.

Antimycin A toxicity: Fe^{3+} effects on PTS cytotoxicity, lipid peroxidation, and adenine nucleotide concentrations. To further assess the ability of iron to confer protection against ATP depletion–mediated PTS injury, Fe^{3+} effects against "chemical anoxia" (i.e., mitochondrial inhibition in the presence of O_2) was assessed. To this end, the influence of the Fe^{3+} on PTS injury induced by antimycin A, a mitochondrial respiratory chain inhibitor, was determined. Five PTS preparations were used for this study. Each preparation was divided into four aliquots: control incubation; antimycin A incubation, 7.5 μM; 2 mM Fe^{3+} addition; and antimycin + 2 mM Fe^{3+} addition. Antimycin A (Sigma Chemical Co., St. Louis, MO) was prepared as a 250× ethanol stock solution, 10 μl of the stock being added to aliquots 2 and 4. To control for possible independent ethanol effects, 10 μl of ethanol (no antimycin) was added to aliquots 1 and 3. After 45 min of incubation (each conducted in the presence of 95% O_2/5% CO_2), %LDH release, MDA, and adenine nucleotide concentrations were determined.

Calculations and statistics. All values are given as mean±SEM. Overall statistical comparisons were performed by one-way analysis of variance for repeated measures. Specific comparisons were made by

paired Student's t test, the Bonferroni correction being applied for multiple comparisons. The %LDH release data reported are the absolute values obtained, i.e., without subtraction of the baseline percent of LDH released (5±1% for all experiments). Statistical significance was judged by a P value of < 0.05.

Results

Effects of Fe^{2+} on PTS viability in the presence and absence of hypoxic-reoxygenation injury. As depicted in Fig. 1 *A*, Fe^{2+} addition to continuously oxygenated PTS caused significant cytotoxicity, 31±2% LDH release being observed after 42 min of incubation ($P < 0.001$ vs. time-matched oxygenated control of 9±1%). Hypoxia-reoxygenation also caused substantial cell killing, 36±2% of LDH being released by the end of the protocol (Fig. 1 *A*). Despite the fact that Fe^{2+} and hypoxia-reoxygenation each induced substantial cell killing (31 and 36% LDH release, respectively), the combination of Fe^{2+} plus hypoxia/reoxygenation produced only 41±2% LDH release, far less than the sum of their independent effects (67%). Of note, a lack of more substantial LDH release in these experiments could not be attributed to a "ceiling" effect (i.e., only 41% of cells could be killed), since in pilot studies performed at this time it was found that prolonged hypoxia (50 min) induced 56, 64, and 65% LDH release, in three PTS preparations so tested.

To more fully explore why Fe^{2+} plus hypoxia-reoxygenation induced far less LDH release than the sum of their independent injurous effects, %LDH release was assessed after hypoxia *without* reoxygenation, both in the presence and absence of Fe^{2+}. As depicted in Fig. 1 *B*, Fe^{2+} + hypoxia induced significantly less LDH release (24±3%) than did hypoxia alone (31±3%; $P < 0.002$), indicating that Fe^{2+} mitigated the hypoxic injury phase. This was despite the fact that Fe^{2+} induced significant cytotoxicity within this 27-min time frame when maintained under oxygenated conditions (19±2 vs. 8±1% for non-iron–exposed controls).

Fe^{2+}-mediated lipid peroxidation in the presence and absence of hypoxia-reoxygenation. As depicted in Fig. 2 *A*, hypoxia in the absence of Fe^{2+} halved MDA concentrations compared with coincubated oxygenated controls (0.4±0.03 vs. 0.8±0.01 nmol/mg protein; $P < 0.05$). Fe^{2+}, in the presence of O_2, raised MDA concentrations to 7.6±0.1 nmol/mg protein. In contrast to the non-iron–exposed PTS (in which hypoxia decreased MDA concentrations), in the presence of Fe^{2+}, hypoxia dramatically increased lipid peroxidation, raising MDA values to 12.7±0.5 nmol/mg protein ($P < 0.01$ vs. iron incubation alone).

As shown in Fig. 2 *B*, after reoxygenation, the previously hypoxic/non-iron–exposed PTS still had significantly lower MDA values than their continuously oxygenated controls (0.4±0.04 vs. 0.6±0.05; $P < 0.03$) (indicating that hypoxia-reoxygenation decreased, rather than increased lipid peroxidation). Comparable MDA values were observed in the Fe^{2+}-exposed PTS aliquots whether they had been subjected to hypoxia-reoxygenation or to continuous oxygenated incubation (indicating that the greater MDA increments observed during Fe^{2+}/hypoxic incubation vs. Fe^{2+}/oxygenated incubation were dissipated during the reoxygenation period).

Fe^{3+} effects on PTS viability and lipid peroxidation in the presence and absence of hypoxia-reoxygenation. As depicted in Fig. 3 *A*, incubating PTS with Fe^{3+} for 42 min under continuously oxygenated conditions induced no cytotoxicity (9±1%

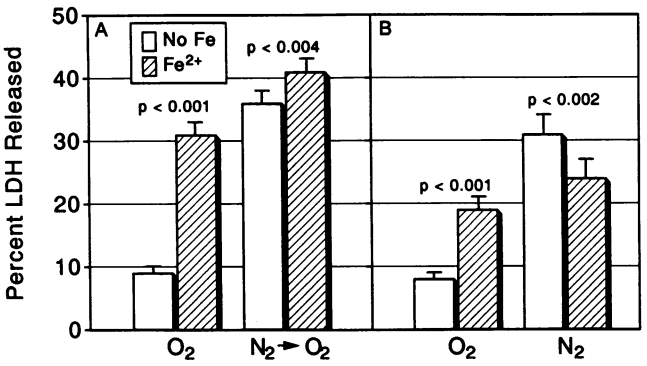

Figure 1. (*A*) Percent LDH released after either 42 min of continuous oxygenation (O_2) or after 27 min of hypoxia plus 15 min of reoxygenation ($N_2 \rightarrow O_2$). Despite the fact that Fe^{2+} and hypoxia-reoxygenation each caused marked cell injury (31 and 36% LDH release, respectively), together only 41% LDH release resulted ($n = 13$ sets of observations). (*B*) Percent LDH released during 27 min of either oxygenated (O_2) or hypoxic (N_2) incubation in the presence or absence of Fe^{2+}. Despite the fact that Fe^{2+} caused LDH release under oxygenated conditions ($P < 0.001$ vs. non-iron–exposed controls), it decreased LDH release during hypoxia ($P < 0.002$ vs. non-iron–exposed controls; $n = 8$ sets of observations).

LDH release for both Fe^{3+} and control PTS). Fe^{3+} exerted a clear cytoprotective effect vs. hypoxic-reoxygenation injury, decreasing %LDH release from 39±2 to 25±2% by the end of the 42-min protocol ($P < 0.002$). To more accurately reflect the degree to which Fe^{3+} had mitigated hypoxia-reoxygenation–induced cell killing, the background cell injury (%LDH released after 42 min of oxygenated incubation; 9±1%) was subtracted from the total %LDH released after hypoxia-reoxygenation in the absence (39±2%) and presence (25±2%) of Fe^{3+}. By so doing, hypoxic-reoxygenation–specific cell injury

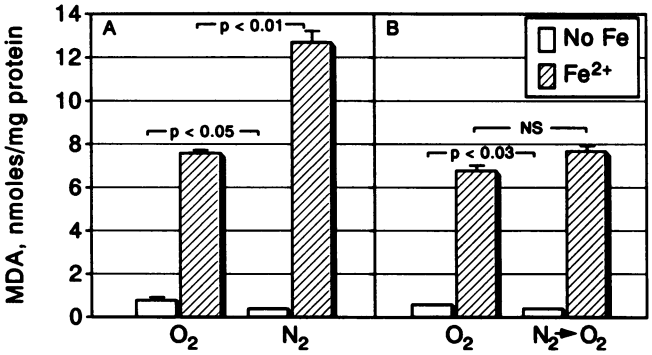

Figure 2. (*A*) MDA concentrations after 27 min of oxygenated (O_2) or hypoxic (N_2) incubations in the presence or absence of Fe^{2+}. In the absence of Fe^{2+}, hypoxia decreased MDA ($P < 0.05$ vs. oxygenated controls). Fe^{2+} caused a dramatic MDA increment, the degree of which was almost doubled by hypoxic compared with oxygenated incubation ($P < 0.01$). (*B*) MDA values after 42 min of oxygenation (O_2) or after 27 min of hypoxia plus 15 min of reoxygenation ($N_2 \rightarrow O_2$) in the presence or absence of Fe^{2+}. Hypoxia-reoxygenation (no Fe^{2+}) caused significantly less MDA generation than did continuous oxygenated incubation ($P < 0.03$). Fe^{2+} caused comparable MDA elevations whether subjected to the O_2 or $N_2 \rightarrow O_2$ incubations. (Thus, the hypoxia-induced increment in Fe^{2+}-mediated lipid peroxidation [*A*] was eliminated during reoxygenation, suggesting lipid peroxide metabolism during reoxygenation.)

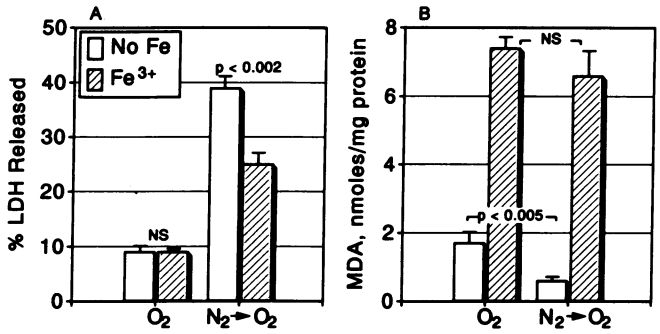

Figure 3. (*A*) Percent LDH released after either 42 min of oxygenated incubation (O_2) or 27 min of hypoxia + 15 min of reoxygenation ($N_2 \rightarrow O_2$) in the presence or absence of Fe^{3+}. Fe^{3+} caused no LDH release and it significantly decreased the LDH release caused by the hypoxia-roxygenation protocol. (*B*) MDA generated by the above protocol. The $N_2 \rightarrow O_2$ protocol (no Fe^{3+}) decreased MDA production compared with continuous oxygenation ($P < 0.005$). However, the Fe^{3+}-driven MDA increments did not significantly differ (analogous to the Fig. 2 *B* results).

could be determined and it was approximately halved by the Fe^{3+} addition (30±2 vs. 16±2%; $P < 0.005$).

MDA concentrations for these experiments are depicted in Fig. 3 *B*. Confirming the observations shown in Fig. 2 *B*, hypoxia-reoxygenation in the absence of iron significantly decreased MDA concentrations compared with continuously oxygenated controls ($P < 0.005$). Fe^{3+} induced marked lipid peroxidation, despite the fact that it induced no cytotoxicity and protected against hypoxia-reoxygenation. Hypoxia-reoxygenation did not alter the iron-driven MDA increments, determined upon the completion of the 42-min experiments (consistent with Fig. 2 *B*).

Iron effects on PTS adenine nucleotide profiles in the presence and absence of hypoxia. Fig. 4 *A* depicts the %LDH released from the PTS aliquots used for analyzing adenylate profiles. After 27 min of oxygenated incubation, Fe^{2+}, but not Fe^{3+}, caused significant cytotoxicity. Hypoxic incubation caused 31±2% LDH release. Both Fe^{2+} and Fe^{3+} mitigated this injury and approximately to the same extent (23±2 and 21±3% LDH release, respectively; both $P < 0.001$ vs. hypoxia alone).

Under oxygenated conditions, both Fe^{2+} and Fe^{3+} induced ~ 25% ATP decrements compared with the non-iron–exposed controls (Fig. 4 *B*). Fe^{2+} and Fe^{3+} also significantly worsened hypoxia-induced ATP depletion (ATP concentrations: hypoxia, 1.4±0.06; hypoxia + Fe^{3+}, 0.9±0.09; hypoxia + Fe^{2+}, 0.4±0.04 nmol/mg protein; all significantly different) despite the fact that Fe^{2+} and Fe^{3+} each conferred cytoprotection, as noted above. The loss of ATP in these experiments was also expressed in decrements of the total adenine nucleotide pool (ATP + ADP + AMP: controls, 7.39±0.52; Fe^{2+}/O_2, 5.67±0.26; Fe^{3+}/O_2, 5.86±0.31; N_2 alone, 2.62±0.16; N_2/Fe^{2+}, 2.22±0.19; N_2/Fe^{3+}, 2.19±0.19).

Effect of Fe/DFO on hypoxic-reoxygenation injury. Fe^{2+} + DFO conferred protection against the hypoxic-reoxygenation protocol (hypoxia-reoxygenation alone, 38±2% LDH release; hypoxia-reoxygenation + Fe^{2+} + DFO, 23±3%; $P < 0.002$). Of note, this protection could not be attributed to DFO alone since it by itself did not alter the hypoxic-reoxygenation injury (37±2% LDH release). The DFO addition largely but not completely blocked the iron-mediated MDA increments [Fe^{2+} alone, 7.6±0.1 (from Fig. 2); Fe + DFO, 1.0±0.1; DFO alone, 0.5±0.06; no addition, 0.6±0.07].

Fe^{3+} effects on antimycin A–mediated injury. As depicted in Fig. 5, antimycin A induced significant but variable cytotoxicity (overall, 41±4% LDH release; individual values of 31, 36, 39, 46, and 51%). Fe^{3+} addition mitigated this injury in every instance (to 17, 23, 21, 32, and 33%, respectively, overall 25±3%; $P < 0.001$). This protective effect occurred despite marked Fe^{3+}-mediated lipid peroxidation (MDA values: antimycin + Fe^{3+}, 5.8±0.5; antimycin alone, 0.7±0.1). As with

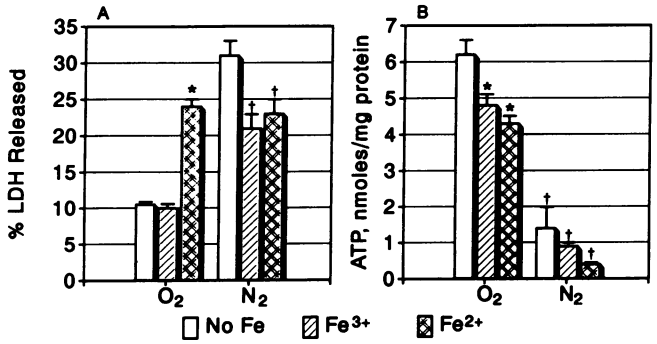

Figure 4. (*A*) Depiction of LDH release from those PTSs used for adenine nucleotide analysis. As noted previously, Fe^{2+} in the presence of O_2 caused significant LDH release compared with the control and Fe^{3+} exposed aliquots (* = $P < 0.002$). Both Fe^{2+} and Fe^{3+} blunted hypoxic (N_2) LDH release and approximately to the same degree († = $P < 0.01$ vs. N_2/no iron exposure). (*B*) ATP concentrations in the above described PTS aliquots. Under oxygenated conditions, Fe^{2+} and Fe^{3+} each lowered ATP concentrations (* = $P < 0.01$ vs. oxygenated controls). Despite the fact that Fe^{2+} and Fe^{3+} each blunted hypoxic LDH release, each worsened the extent of ATP depletion († = all three significantly different, $P < 0.05$).

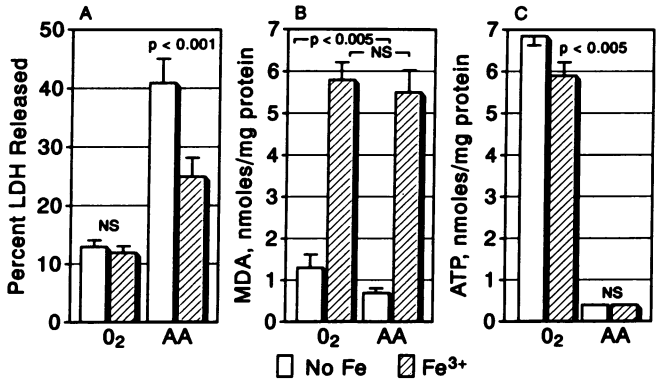

Figure 5. (*A*) LDH release under oxygenated conditions±antimycin A (*AA*), assessed after 45 min of incubation. Fe^{3+} substantially decreased AA-induced LDH release ($P < 0.001$). (If one subtracts out the background LDH release [i.e., in the absence of AA], then Fe^{3+} decreased AA cell killing by ~ 50%.) (*B*) MDA concentrations from the above experiments. AA significantly decreased MDA in the absence of Fe^{3+} ($P < 0.005$). However, AA did not alter the iron-driven MDA increments. (*C*) ATP concentrations in the above experiments. Fe^{3+} caused a modest decrease in ATP in the absence of AA ($P < 0.005$). Profound ATP depletion was induced by AA, the degree of which was not altered by concomitant Fe^{3+} exposure.

hypoxia, antimycin A in the absence of iron approximately halved MDA concentrations. However, unlike the hypoxia experiments, antimycin did not increase MDA concentrations in the presence of iron. Antimycin induced profound ATP depletion (from 6.85±0.2 to 0.37±0.01 nmol/mg protein), a result that was not altered by Fe^{3+} addition (0.36±0.01) (indicating that iron did not block the bioavailability of antimycin).

Discussion

It has been widely proposed that oxygen free radical formation (e.g., from xanthine oxidase activity) with resultant oxidant tissue stress is a critical mediator of ischemic-reperfusion injury of multiple organs (16, 22, 23), including the kidney (e.g., reference 24). It has also been suggested that free (nonprotein-bound) iron can dramatically accentuate this pathway of injury, both by directly participating in free radical formation (e.g., ferryl/perferryl ion), and by catalyzing the conversion of H_2O_2 to highly toxic hydroxyl radical via the Fenton/Haber-Weiss reactions (11, 16, 25–29). Recent experiments from this laboratory illustrate this latter point. When BSA was incubated with either $FeSO_4$ or H_2O_2, modest protein oxidation was induced (denoted by ~ 35% increments in the carbonyl content of BSA) (10). However, when BSA was exposed to H_2O_2 and Fe^{2+} simultaneously, a 6,000% carbonyl content increment resulted (unpublished data). Thus, it is clear that iron can indeed dramatically potentiate oxidative tissue reactions. A recent study by Paller and Hedlund (11) suggests that a similar phenomenon may occur in the in vivo kidney. By studying the renal arterial occlusion model of ARF, they observed that $FeCl_3$–EDTA infusion worsened, whereas DFO administration protected against, ischemic injury (11). That $FeCl_3$ increased and DFO decreased renal cortical lipid peroxidation (assessed by MDA) suggested that oxidant mechanisms were operative. The potential relevance of these in vivo observations extends beyond the renal artery occlusion model of ARF. For example, during the initiation phase of myohemoglobinuric ARF, catalytic (free) iron is generated (10), it gains access to tubular lumina (10), and concomitant renal ischemia develops (8, 30). Thus, the goal of the present study was to directly assess at the cellular level the impact of catalytic iron on O_2 deprivation-reoxygenation PTS injury in an effort to better understand mechanisms of myohemoglobinuric as well as ischemic ARF.

To begin these studies, PTSs were subjected to 27 min of hypoxia plus 15 min of reoxygenation either in the presence or absence of a toxic Fe^{2+} concentration. A relatively short hypoxic insult was chosen such that a sufficient number of tubular cells would remain viable, thereby permitting additive/synergistic injury to occur. Despite the fact that hypoxia-reoxygenation alone and Fe^{2+} alone each induced substantial cell killing (31 and 36% LDH release, respectively), the two in combination produced only 41% LDH release (Fig. 1 *A*), suggesting mutually exclusive, not additive/synergistic injury. The simplest possible explanation for this observation would be a "ceiling effect," i.e., that only 41% of cells could be killed, and thus, synergistic injury could not occur. However, both past experience with the PTS model (31, 32) and our present confirmation of > 60% LDH release in response to 50 min of hypoxia exclude this possibility. Thus, the above data indicate that Fe^{2+} and hypoxia-reoxygenation either induce injury via a common, saturable pathway or that one insult induces cellular changes that confer protection against the other.

In an attempt to gain insights into this issue, the impact of Fe^{2+} on both the hypoxic and the reoxygenation injury phases were individually assessed. It was reasoned that if the time sequence of injury could be better defined a clearer understanding of why Fe^{2+} and hypoxia-reoxygenation fail to induce additive injury might result. These experiments indicated the following: Fe^{2+} significantly blunted the cell injury that resulted from 27 min of hypoxic incubation; this protective effect occurred despite the fact that 27 min was sufficient time for Fe^{2+}, under oxygenated conditions, to exert its own cytotoxic effect (Fig. 1 *B*); during reoxygenation Fe^{2+} exposed/previously hypoxic PTS experienced a rapid phase of LDH release (increase of 17%); and this latter phenomenon was of sufficient magnitude to cancel out the previous protective influence of iron. Because it is widely held that iron promotes "reperfusion" injury, it is tempting to postulate that brisk LDH release during reoxygenation was due to synergistic oxidant tissue stress. However, it is important to note that this 17% LDH release exactly equalled the sum of independent Fe^{2+} (12%) and reoxygenation (5%) effects (defined by the time-matched single insult PTS aliquots). This fact, plus falling rather than rising MDA concentrations during this period (Fig. 2), strongly suggest that this reoxygenation phase was not one of synergistic oxidant tissue injury. In summary, these time frame experiments indicate that, overall, Fe^{2+} and hypoxia-reoxygenation do not induce fully additive or synergistic injury because Fe^{2+} mitigates the hypoxic injury phase.

Although the above experiments demonstrated that Fe^{2+} and hypoxia-reoxygenation do not necessarily cause additive/synergistic injury, the conclusion that Fe^{2+} actually mitigates O_2 deprivation injury might be questioned because the observed protection was only transient, not extending beyond the hypoxic period. Thus, in an attempt to substantiate that iron can indeed confer significant and durable protection, additional experiments were undertaken using Fe^{3+} as the iron challenge. The rationale for this was that since Fe^{3+} has no independent cytotoxic effect it, like Fe^{2+}, might be able to blunt hypoxic injury but it would not induce cell killing during the reoxygenation period. In addition, the ability of Fe^{3+} to block antimycin A–mediated injury was assessed, both to further substantiate the protective influence of iron and to discern whether this protection can be expressed in the presence of O_2, which theoretically permits maximal free radical formation rates. These experiments yielded the following significant results: Fe^{3+} approximately halved hypoxic injury, whether assessed at the end of hypoxia or after the reoxygenation phase (indicating durable protection); Fe^{3+} induced its cytoprotective influence *despite* evoking marked MDA generation; and Fe^{3+} markedly attenuated antimycin A–induced cell killing, thereby substantiating its protective influence and proving that it can express itself despite high O_2 tensions.

Although the above findings seemingly challenge the prevailing view that catalytic iron enhances "ischemic-reperfusion" injury (e.g., references 22–26), a number of potentially important caveats need to be borne in mind before extrapolating these results to the in vivo situation. First, a variety of different mechanisms may be operative in "hypoxic-reoxygenation" vs. "ischemic-reperfusion" injury. Second, although isolated PTSs are widely used as an in vitro model for acute tubular injury, their relevance to in vivo ARF remains to be proven; and third, to induce cell injury and lipid peroxidation in this in vitro system, millimolar ion concentrations were re-

quired, presumably because of limited iron uptake by tubular cells. Thus, results obtained with these large iron challenges may have limited pathophysiological relevance. However, recent experiments from this laboratory suggest that this concentration issue may not in fact be critical. It was observed that when PTSs were prepared without DFO addition to the collagenase digestion buffer, ~ 2–10 μM PTS iron loading results (32). When such PTSs were incubated under oxygenated conditions, they had limited viability because of the resulting iron toxicity (33±1% LDH over 60 min) (19). However, when these iron-damaged PTSs were challenged with 45 min of hypoxia + 15 min of reoxygenation, minimal additional cell killing occurred (11% increment over their oxygenated counterparts vs. a 46% increment in non–iron-loaded PTS) (19). Thus, these results (which provided the impetus for the current study) strongly support the conclusion that iron, even in micromolar concentrations, can blunt O_2 deprivation–mediated PTS injury.

Having confirmed that Fe^{2+} and Fe^{3+} can each mitigate hypoxic renal injury, we next addressed whether this protection was mediated via an improvement in cellular energetics. Thus, Fe^{2+}/Fe^{3+} effects on adenine nucleotide pools were assessed in the presence and absence of hypoxia. Despite the fact that both iron moieties attenuated hypoxic LDH release, they each exacerbated rather than improved the extent of ATP depletion. Thus, iron protected against hypoxia by mitigating the consequences of ATP depletion rather than the severity of it. That Fe^{3+} also lessened antimycin A toxicity without improving adenylate pools further supports this conclusion. Of additional interest were findings that Fe^{2+} and Fe^{3+} each caused ~ 25% decrements in ATP and total adenine nucleotide concentrations under oxygenated conditions. Thus, these data support previous in vivo results suggesting that iron lowers cortical ATP concentrations by ~ 25% independent of changes in renal hemodynamics (18). The mechanism for this iron-mediated adenylate loss remains unknown. However, that iron is capable of directly chelating adenine nucleotides (27) and that cell membrane lipid peroxidation might secondarily cause adenine nucleotide depletion are two possibilities. Given that iron causes ATP decrements in the absence of hypoxia, it is not surprising that the degree of ATP depletion during hypoxia was also exacerbated by iron exposure.

The final goal of this study was to assess the impact hypoxia-reoxygenation on proximal tubular lipid peroxidation both in the presence and absence of catalytic iron. Specifically, the following two questions were addressed: does hypoxia-reoxygenation cause MDA generation, suggesting a period of rapid free radical formation? and what impact does hypoxia-reoxygenation have on iron-driven MDA increments? As depicted in Fig. 2, hypoxia not unexpectedly decreased MDA generation by non-iron–exposed PTS, suggesting that O_2 deprivation limits oxygen free radical formation. However, during reoxygenation, no rebound lipid peroxidation resulted, MDA concentrations remaining significantly lower than those found in the continuously oxygenated controls. Thus, these data support a growing body of evidence that indicates that O_2 deprivation-reoxygenation PTS injury is not oxygen free radical/lipid peroxidation dependent (19, 32–34). Paradoxically, hypoxia appeared to double the amount of iron-mediated lipid peroxidation, at least as assessed by the MDA assay. The reason for this surprising result is unknown. Since MDA levels undoubtedly reflect a balance between lipid peroxide synthesis and mitochondrial metabolism, one possible explanation is that sufficient O_2 is present during hypoxia to permit the former, but not the latter, to occur. However, that antimycin A did not also induce an increment in iron-driven MDA generation speaks against this possibility. Thus, an alternative explanation for the greater MDA increments is that Fe^{2+} and hypoxia induce synergistic oxidant stress via the Fenton/Haber-Weiss reactions. However, if true, it is important to note that this temporally correlated with cytoprotection, not cell injury. Conversely, during reoxygenation MDA concentrations fell (presumably due to lipid peroxide catabolism) despite the fact that cellular injury resulted. Thus, these results serve to underscore our previous conclusions (15) that MDA generation and critical cell injury may bear little relationship.

The mechanism by which iron confers protection against hypoxic- and antimycin-mediated injury remains to be defined. One intriguing possibility is that iron-induced lipid peroxidation, rather than being injurious, might paradoxically confer a protective effect. To test this possibility, the influence of iron + DFO on hypoxia-reoxygenation injury was assessed. Since excess DFO can block iron-mediated lipid peroxidation, if iron + DFO were able to confer protection, it would serve to dissociate iron-induced lipid peroxidation from its cytoprotective effect. This appeared to be the case since iron + DFO substantially reduced hypoxic-reoxygenation injury despite trivial MDA generation. Of note, this cytoprotective influence cannot be explained by a pure DFO effect since both past (32, 34) and present data indicate that DFO does not by itself protect PTS from hypoxic-reoxygenation injury.

Lastly, although the present experiments were directed at exploring the nature of iron–hypoxic interactions, they also provide two new insights into determinants of Fe^{2+}-induced cytotoxicity. First, that Fe^{2+} appeared to cause no LDH release under hypoxic conditions (Fig. 4) indicates that Fe^{2+} cytotoxicity is O_2 dependent. Although this suggests that Fe^{2+} toxicity is due to an oxygen free radical–dependent reaction, the finding of greater Fe^{2+}-mediated MDA elevations during Fe^{2+}/hypoxic incubation versus Fe^{2+}/oxygenated incubation (Fig. 2 *A*) argues against this hypothesis. Thus, an alternative possibility is that hypoxia precludes the expression of Fe^{2+} toxicity because the resulting ATP depletion prevents Fe^{2+} transport to critical intracellular targets. Although this remains a hypothesis, it does point out that an O_2 requirement for the expression of Fe^{2+} toxicity does not necessarily equate with a free radical–based reaction. Second, the present study demonstrates for the first time that Fe^{2+}-induced PTS toxicity is not mediated by ATP depletion. This conclusion is based on findings that 27 min of Fe^{2+} exposure induced LDH release despite only modest ATP reductions (~ 25%) and that Fe^{2+} and Fe^{3+} caused comparable ATP losses and yet only the former caused LDH release.

In conclusion, the present study indicates that cytotoxic Fe^{2+} can blunt hypoxic PTS injury despite inducing lipid peroxidation and lowering ATP. However, this beneficial effect is transitory because during reoxygenation Fe^{2+} is able to express its intrinsic cytotoxic effect. Fe^{3+}, like Fe^{2+}, also blunts hypoxic PTS injury. However, unlike Fe^{2+}, Fe^{3+}-mediated protection persists throughout reoxygenation because it exerts no intrinsic cytotoxicity. Fe^{3+} also protects against antimycin A–mediated PTS injury independent of changes in cellular energetics. This confirms that iron can indeed blunt ATP depletion–mediated injury and that this protective influence is not dependent on

concomitant hypoxia (which theoretically could limit oxygen free radical generation); and hypoxia and antimycin A inhibit PTS lipid peroxide formation. However, hypoxia paradoxically increases iron-driven MDA increments, possibly by creating the necessary conditions for the Fenton/Haber-Weiss reactions. In sum, although these experiments indicate that iron may promote lipid peroxidation during hypoxia, cytoprotection rather than additive/synergistic injury can result. Thus, the ability of heme proteins to exacerbate in vivo ischemic tubular injury cannot simply be explained by a direct iron–ischemic interaction. Previous observations from this laboratory that both iron- and non-iron–containing filtered proteins can equally exacerbate experimental ischemic renal injury further support this view (6).

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