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Role of iron in postischemic renal injury in the rat

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Role of iron in postischemic renal injury in the rat. To determine whether iron participates in free radical-mediated postischemic renal injury and lipid peroxidation, we examined the effects of removal of endogenous iron or provision of exogenous iron following renal ischemia, as well as the effects of renal ischemia and reperfusion on renal venous and urinary "free" iron. Rats underwent 60 minutes of renal ischemia and were studied after either 24 hours (inulin clearance) or 15 minutes (renal malondialdehyde content) of reperfusion. Infusion of the iron chelator deferoxamine (200 mg/kg/hr) during the first 60 minutes of reperfusion resulted in a marked improvement in renal function (inulin clearance: 879 ± 154 vs. 314 ± 74 μl/min; $P < 0.025$) and a reduction in lipid peroxidation (renal malondialdehyde: 0.449 ± 0.06 vs. 0.698 ± 0.08 mmol/mg prot; $P < 0.05$) compared to control animals. Infusion of 50 mg/kg/hr deferoxamine also protected renal function after ischemia (inulin clearance: 624 ± 116 vs. 285 ± 90 μl/min; $P < 0.05$) and resulted in less histologic injury. Iron-saturated deferoxamine had no protective effect. Conversely, infusion of the iron complex EDTA-$FeCl_3$ during reperfusion exacerbated postischemic renal dysfunction and lipid peroxidation. Following renal ischemia there was no detectable increase in "free" iron in arterial or renal venous plasma. However, urinary "free" iron increased 10- to 20-fold following reperfusion. Iron chelators which underwent filtration and gained access to this free iron in the urine (free deferoxamine or inulin-conjugated deferoxamine) provided protection, whereas a chelator confined to the vascular space (dextran-conjugated deferoxamine) did not. We propose that during ischemia and reperfusion iron is released from storage pools and is available to catalyze hydroxyl radical formation and lipid peroxidation. Administration of the iron chelator deferoxamine during reperfusion limits postischemic renal dysfunction and free radical-mediated lipid peroxidation. These effects appear to take place in the urinary space or along the brush border membrane adjacent to the urinary space.

Oxygen free radicals are now well recognized to contribute to kidney damage after ischemia and reperfusion [1–5]. During ischemia ATP is dephosphorylated and the adenosine is further degraded. During reperfusion, when xanthine oxidase converts hypoxanthine to xanthine, an electron is transferred from hypoxanthine to molecular oxygen to produce the oxygen free radical superoxide radical (O_2^-). Superoxide radical is further metabolized to hydrogen peroxide and hydroxyl radical (OH ·). All three reactive oxygen species have toxicity, but hydroxyl radical is the most highly reactive [1].

The toxicity of reactive oxygen species is a consequence of their ability to react with proteins, lipids, polysaccharides, and DNA [6, 7]. Essential enzymes with sulfhydryl groups can be inactivated when oxidized. Peroxidation of polyunsaturated fatty acids makes membranes more permeable and can interfere with mitochondrial and cellular function. Depolymerization of polysaccharides also affects cellular function, whereas degradation of DNA could interfere with postischemic repair which requires cell division.

Some transition metals, in particular iron and copper, catalyze the reaction shown here whereby superoxide radical and hydrogen peroxide yield hydroxyl radical [6]. This net reaction is known as the Haber-Weiss reaction. This reaction can be separated into two steps:

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (1)$$

$$H_2O_2 + Fe^{2+} \rightarrow OH \cdot + OH^- + Fe^{3+} \quad (2)$$

In the first step ferric iron is reduced by superoxide radical. In the second step iron reduces hydrogen peroxide to hydroxyl radical (Fenton's reaction). Although iron in tissues is usually tightly bound to ferritin, xanthine oxidase-derived superoxide radical can release iron from ferritin [8–10]. This free iron can then participate in the formation of hydroxyl radical. In addition iron might directly induce lipid peroxidation [11, 12].

The purpose of this study was to determine the role of iron in postischemic renal injury because of the postulated role of iron in catalyzing hydroxyl radical formation and lipid peroxidation. We sought to determine whether removal of endogenous iron would attenuate renal injury after ischemia and whether provision of exogenous iron would exacerbate postischemic injury. We also sought to determine whether there were detectable changes in free iron in either plasma or urine following ischemia and perfusion. Finally, we sought to determine the nephron site where iron chelators were protective against postischemic injury.

Methods

Model of ischemic renal failure

Male Sprague-Dawley rats (Harlan, Madison, Wisconsin, USA) weighing 225 to 275 g were used. They were allowed free access to food and water until the time of study. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.), and a femoral vein catheter (PE-50) inserted for administration of drugs. Bilateral flank incisions were made and the right kidney was removed. The left kidney was exposed, the perirenal fat removed, and the left renal artery exposed. Heparin (100 IU/kg,

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i.v.) was administered, and a non-traumatic vascular clamp was then placed across the renal artery for 60 minutes. After removal of the clamp the animal was sutured and allowed to recover for study 24 hours later.

The effects of renal ischemia on renal function were assessed by measurement of inulin clearance 24 hours after renal ischemia. Animals were anesthetized with pentobarbital and placed on a heated, temperature-controlled table. Catheters were placed in the femoral artery and vein, and ureter. An infusion of 10% inulin in saline was begun at 0.06 ml/min for 15 minutes, followed by 0.02 ml/min for the duration of the study. After a 60 minute equilibration period, three 20 minute urine collections were made. Blood for plasma inulin determination was obtained at the midpoint of each collection. The concentration of inulin in urine and blood was determined by the anthrone method and inulin clearance was calculated by the standard formula.

Lipid peroxidation was measured after a 60 minute ischemia plus 15 minute reperfusion, a time of maximum lipid peroxidation [2, 3] by determining the renal cortical content of the lipid peroxidation product malondialdehyde (MDA). MDA was measured by the method of Ohkawa, Ohishi and Yagi [13]. After ischemia plus reflow, kidneys were rapidly removed and placed in iced phosphate buffered saline. Sections of renal cortex were suspended in a total volume of 3 ml 100 mM KCl plus 0.003 M EDTA and homogenized with a Polytron (Brinkman Instruments, Wesbury, New York, USA) at setting 8 for 15 seconds. Homogenates were then centrifuged at 600 g for 10 minutes. Two hundred microliters of supernate were added to 0.2 ml 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (pH 3.5), 1.5 ml 0.8% thiobarbituric acid and 6.6 ml water. This solution was heated to 95°C for 60 minutes. After addition of 1.0 ml water and 5.0 ml of an n-butanol/pyridine mixture (15:1 vol/vol) the mixture was vigorously shaken and centrifuged at 2000 g for 15 minutes. The absorbance of the upper organic layer at 532 nm was determined in a spectrophotometer. Absorbance of tissue samples was compared to results obtained using malonaldehyde tetraethylacetal standards (Sigma Chemical Co., St. Louis, Missouri, USA). MDA values were expressed per mg protein. All determinations of MDA were performed in duplicate.

Removal of endogenous iron

To remove endogenous iron after ischemia, the iron chelator deferoxamine (deferoxamine mesylate, Ciba Pharmaceutical Co., Summit, New Jersey, USA) was infused during the first hour of reperfusion. In the first set of studies deferoxamine (50 mg/ml in 5% dextrose) was infused at the rate of 200 mg/kg/hr beginning one minute before removal of the renal artery clamp and continuing for 60 minutes. Inulin clearance was determined 24 hours later in six rats. Control rats were treated similarly except that they received an infusion of 5% dextrose of 1 ml/hr. To ascertain that any beneficial effect of deferoxamine was due to its ability to chelate iron, an additional group of animals was infused with iron-saturated deferoxamine. Iron-saturated deferoxamine was produced by mixing equimolar amounts of deferoxamine and $FeCl_3$ and infused to deliver a dose containing an amount of deferoxamine of 200 mg/kg/hr for the first hour of reperfusion. The effect of iron-saturated deferoxamine on postischemic renal function was determined by measuring inulin clearance 24 hours later. In other studies deferoxamine was given in a dose of 50 mg/kg/hr for one hour and the effect on inulin clearance determined 24 hours later. In another group of rats deferoxamine or the dextrose vehicle was given at the same rate during the first 15 minutes of reperfusion. The animals were then sacrificed and their kidneys removed for determination of malondialdehyde content.

The systemic and renal hemodynamic effects of deferoxamine in normal rats was also determined. Animals were prepared for inulin clearance studies as described above. In addition a 23 gauge needle was inserted into the left renal vein so that samples of renal vein blood could be removed to determine the renal extraction of inulin and calculate renal plasma flow. Two 20-minute collections were performed, then an infusion of deferoxamine was begun in a dose of 200 mg/kg/hr. After 10 minutes two additional clearance periods were performed.

Effect of iron infusion

To attempt to increase iron availability after ischemia, the iron complex EDTA-$FeCl_3$ was infused during the first five minutes of reperfusion in a dose of 24 nmol/kg [14, 15]. Ferric-EDTA was prepared by mixing equimolar amounts of Na_2H_2 EDTA and $FeCl_3$ (12 μM)in saline [16]. Control animals received EDTA in saline (0.5 ml) during the first five minutes of reperfusion. A second control group received 5% dextrose during the first five minutes of reperfusion.

Morphologic studies

Kidneys from control animals (N = 6) and deferoxamine-infused animals (N = 6; 50 mg/kg/hr) were examined by light microscopy. After ischemia and 24 hours of reflow, kidneys were fixed by perfusion at mean arterial pressure with 1.25% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4, osmolality 300 mOsm/kg), followed by immersion in fixative for three hours. Sections were embedded in paraffin and stained with hematoxylin and eosin. Slides were coded by a technician not involved in this study, and reviewed in a blinded manner and scored by one of us (M.S.P.) using a semiquantitative scale to evaluate the presence and extent of tubular epithelial cell flattening, brush border loss, cell membrane bleb formation, cytoplasmic vacuolization, cell necrosis, interstitial edema and tubular lumen obstruction [2]. For each kidney, 50 cortical tubules from at least 10 different areas were scored with care taken to avoid repeated scoring of different convolutions of the same tubule. Higher scores represent more severe damage (maximum score per tubule was ten points).

Effect of ischemia and reperfusion on iron availability

Plasma and urine samples were analyzed for "catalytic" iron using an assay developed by Gutteridge, Rowley and Halliwell [17, 18]. In this assay trace amounts of free (non-protein bound) iron capable of participating in superoxide-dependent hydroxyl radical formation can be detected by measuring the degradation of DNA by bleomycin, a reaction which requires the presence of Fe^{2+}.

All reagents were prepared in pyrogen-free water treated by distillation, reverse osmosis, and shaking with Chelex-100 (Biorad Laboratories, Richmond, California, USA) to remove contaminating iron using new sterile plastic tubes. Bleomycin sulfate (Bristol-Myers Company) was prepared as a 1 mg/ml

solution. Calf thymus DNA (Sigma) was also prepared as a 1 mg/ml solution. The reaction mixture contained 125 μl DNA, 12.5 μl bleomycin, 25 μl $MgSO_4$ (50 mM), 25 μl sample, 12.5 μl HCl (10 mM), 25 μl H_2O and 25 μl fresh ascorbic acid. After mixing, the tubes were incubated at 37°C for two hours. To stop the reaction 250 μl EDTA (100 mM) was added. The content of each tube was transferred to a 12 × 75 polypropylene tube to which 250 μl thiobarbuturic acid (1% wt/vol in 50 mM NaOH) and 250 μl HCl (25% vol/vol) were added. After heating to 100°C for 15 minutes, the tubes were cooled and the absorbance of the solution measured at 532 nm. A standard curve was prepared for each assay with $FeCl_3$ dissolved in chelex-treated pyrogen free water with HCl (10 mM) added to adjust pH to 7.3.

To determine the effect of renal ischemia on plasma free iron, carotid artery and renal vein blood samples were obtained before ischemia, at the end of 60 minutes of ischemia and following 10, 30, and 60 minutes of reperfusion. Urine was collected for 30 minute periods before ischemia, between 15 and 45 minutes of reperfusion (urine output in the first 15 min of reperfusion was often negligible), and between 45 and 75 minutes of reperfusion.

Nephron site of protection by iron chelators

Studies were undertaken to determine whether the free iron released into the urinary space was the critical iron pool responsible for promotion of free radical formation and renal injury during reperfusion. Following 60 minutes of ischemia one of three deferoxamine compounds, or an appropriate vehicle, was infused during the first hour of reperfusion. Inulin clearance was measured 24 hours later, as described above. The three iron chelating compounds were free deferoxamine and deferoxamine conjugated to biocompatible polysaccharides, either inulin (Sigma) or dextran T-40 (Rheomacrodex, Pharmacia, Uppsala, Sweden). The latter two compounds were supplied by Biomedical Frontiers (Minneapolis, Minnesota). Briefly, the compounds were synthesized by oxidizing the polysaccharide with periodate, and allowing the resulting dialdehydes to form a Schiff base with the free amino group of the terminal lysine amino group of deferoxamine. This Schiff base was initially reduced with sodium cyanoborohydride, and remaining unreduced aldehyde groups were subsequently reduced with sodium borohydride. Free deferoxamine and reaction products were removed by extensive dialysis against distilled water. The soluble conjugates were then lyophilized and stored dessicated until use. Iron content of the deferoxamine-polysaccharide conjugates was determined by addition of excess ferrous or ferric iron to stock solutions. Typically, the weight fraction of chelator in these conjugates varied from 10 to 30%. The ability of polysaccharide-bound chelator to bind iron was not altered following incorporation into the polymer matrix (Hallaway PE, Eaton JW, Panter SS, Hedlund BE: unpublished data).

Free deferoxamine has a molecular weight of 560 and a very small effective molecular radius. Following infusion deferoxamine would have access to the blood stream and would then appear in the urinary space after filtration [19]. Deferoxamine might also enter cells, although this point is controversial. Deferoxamine conjugated to inulin has an effective molecular radius of about 14 angstroms. When infused it would have access to the blood and would then appear in the urinary space after filtration [20]. Inulin-conjugated deferoxamine would be excluded from intact cells. Deferoxamine conjugated to dextran has an effective molecular radius of approximately 42 angstroms and would be essentially excluded from glomerular filtration [20] and from cells and would, therefore, have access only to the blood space. Even following ischemia the fractional excretion of dextran-conjugated deferoxamine would not rise substantially to deliver deferoxamine to the urinary space [21].

Deferoxamine was infused in a dose of 50 mg/kg/hr for the first hour of reperfusion. Inulin-conjugated deferoxamine was infused to provide an equivalent amount of deferoxamine. A control group of animals received an equivalent amount of inulin. Dextran-conjugated deferoxamine was given in an amount (230 mg/kg/hr) to yield plasma concentrations during the first hour of reperfusion which would approximate those attained during infusion of a high dose of free deferoxamine of 200 mg/kg/hr. An additional control group received a dextran infusion during the first hour of reperfusion.

Plasma deferoxamine was determined by a spectrophotometric assay for feroxamine after adding saturating quantities of iron to plasma samples. Two hundred μl of plasma and 300 μl of 10 mM $FeSO_4$ were incubated at 25°C for one hour. Five hundred μl of 20% trichloroacetic acid was added, the precipitate removed by centrifugation, and the supernatant mixed with an equal volume of 1 M sodium acetate (pH 5.5). Absorbance at 429 nm was measured before and after iron addition, and the deferoxamine concentration was calculated from the change in feroxamine concentration using a molar extinction coefficient of 2300. Plasma samples for deferoxamine determination were obtained 11, 31, and 61 minutes after the start of infusion of either free deferoxamine or dextran-conjugated deferoxamine.

Statistical analysis

All data are reported as mean ± standard error. Most comparisons were made by Student's *t*-test for unpaired samples. Wilcoxon's rank sum test was used for nonparametric data (effect of ischemia on urinary free iron). To analyze data from the studies of the effect of deferoxamine on hemodynamics in normal rats the paired *t*-test was employed. Analysis of variance, followed by Tukey's test for intergroup comparisons, was used to compare the effect of infusion of deferoxamine, iron-saturated deferoxamine, or dextrose on postischemic GFR, to compare the effect of infusion of EDTA-$FeCl_3$, EDTA, or dextrose on postischemic GFR, and to compare renal malondialdehyde content in nonischemic control kidneys to experimental and control, postischemic kidneys and to compare the effects of the various deferoxamine compounds.

Results

Effect of removal of endogenous iron

Twenty-four hours after 60 minutes of renal ischemia the glomerular filtration rate (GFR) in control rats was 314 ± 74 μl/min ($N = 6$). Animals that had received an infusion of deferoxamine (200 mg/kg/hr) during the first hour of reperfusion were markedly protected against renal dysfunction. In these animals inulin clearance was 879 ± 154 μl/min ($N = 6$; $P < 0.025$). Iron-saturated deferoxamine-treated rats had a postischemic inulin clearance of 433 ± 85 μl/min ($N = 6$), a value not significantly different from dextrose-infused controls and significantly lower than in deferoxamine-treated animals ($P < 0.05$). Deferoxamine also provided protection against postischemic

Table 1. Effect of deferoxamine (200 mg/kg/hr) on hemodynamic renal function in normal rats

	MAP	V	GFR	RPF	RBF	$U_{Na}V$	$U_{Osm}V$
	mm Hg	*μl/min*				*mEq/min*	*μOsm/min*
Baseline	122 ± 7	11.1 ± 2.9	1110 ± 77	2.8 ± 1.0	5.0 ± 0.7	0.30 ± 0.14	13.0 ± 2.2
Deferoxamine	106 ± 8	13.5 ± 3.1	995 ± 37	2.8 ± 0.6	5.0 ± 1.0	0.30 ± 0.09	12.1 ± 2.9
P	<0.05	NS	NS	NS	NS	NS	NS

Values are mean ± SE. Abbreviations are: MAP, mean arterial pressure; V, urine flow rate; GFR, glomerular filtration rate; RPF, renal plasma flow; RBF, renal blood flow; $U_{Na}V$, urinary sodium excretion rate; $U_{Osm}V$, osmolar excretion rate; NS, not significantly different. $N = 5$.

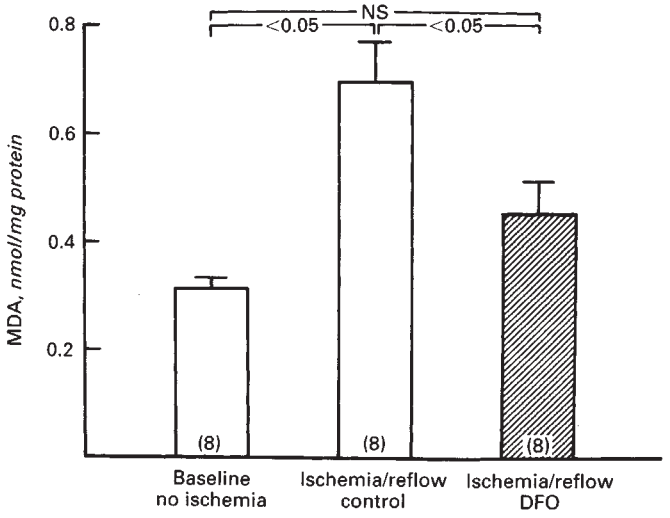

Fig. 1. *Effect of deferoxamine (DFO) on lipid peroxidation after renal ischemia.* Numbers in parentheses represent number of animals studied. NS, not significantly different; MDA, malondialdehyde.

injury when given in a lower dose. In animals given deferoxamine in a dose of 50 mg/kg during the first hour of reperfusion GFR 24 hours later was 624 ± 116 μl/min ($N = 6$). Control animals had a GFR of 285 ± 90 μl/min 24 hours after ischemia ($N = 6$; $P < 0.05$).

Infusion of deferoxamine during reperfusion also reduced postischemic lipid peroxidation (Fig. 1). As can be seen there was a significant increase in renal malondialdehyde content after 60 minutes of ischemia and 15 minutes of reperfusion in control animals. The infusion of deferoxamine during the 15 minute period of reperfusion prevented the increase in renal malondialdehyde content.

This beneficial effect of deferoxamine on postischemic renal function was not likely to be due to a nonspecific effect of the agent. Normal animals infused with the higher dose of deferoxamine (200 mg/kg/hr) showed a significant decrease in mean arterial pressure without any change in urine flow rate, inulin clearance, renal plasma flow, renal blood flow, or sodium or osmolar excretion (Table 1).

Effect of increased iron availability

When iron was administered as the EDTA complex the effects of renal ischemia were exacerbated. Animals infused with 5% dextrose had a GFR of 292 ± 62 μl/min ($N = 6$). Animals infused with EDTA alone had a GFR of 349 ± 81 μl/min ($N = 5$) 24 hours after ischemia. However, when EDTA-$FeCl_3$ was infused during the first five minutes of reperfusion GFR 24 hours later was only 138 ± 28 μl/min ($N = 5$). By analysis of variance there was no difference between the 5% dextrose or EDTA animals. Both of these groups had significantly higher GFR than EDTA-$FeCl_3$-infused animals ($P < 0.05$).

The provision of exogenous iron as EDTA-$FeCl_3$ also caused an increase in lipid peroxidation after ischemia. Renal malondialdehyde content in nonischemic kidneys was 0.476 ± 0.045 nmol/mg prot. In control animals ischemia and 15 minutes of reperfusion resulted in an increase in renal malondialdehyde to 0.614 ± 0.083 nmol/mg prot ($N = 7$), although in this particular experiment the increase did not reach statistical significance when using multiple-group analysis. Animals which had received EDTA-$FeCl_3$ during the first five minutes of reperfusion had a greater increase in renal MDA to 0.781 ± 0.102 nmol/mg prot ($N = 8$; $P < 0.05$ vs. nonischemic kidneys).

Morphologic studies

Kidneys from both groups of animals showed extensive injury. However, this injury was less severe in deferoxamine-treated animals. Casts derived from brush border or sloughed proximal tubular cells were found obstructing the lumens of cortical segments in both groups, but casts in the outer medulla were more frequent in control kidneys and casts in the inner medulla were seen only in control kidneys. Congestion of vasa rectae was also more common in control kidneys. Proximal tubular cells in both groups showed brush border loss and bleb formation. In deferoxamine-treated kidneys, however, extensive cellular necrosis and sloughing of cells were much less often seen than in control kidneys (Figs. 2 and 3). Injury was also less severe in deferoxamine-treated animals when a semiquantitative scoring system was used to score coded slides. Proximal tubules in control kidneys had a mean score of 7.2 ± 0.1, whereas those of deferoxamine-treated kidneys had a mean score of 6.7 ± 0.1 ($P = 0.053$) [22]. Therefore, deferoxamine resulted in only modest histologic protection compared to the more substantial functional protection.

Effect of ischemia on free iron

Free iron was not detected in either arterial or renal venous plasma before, during or following ischemia. However, small amounts of free iron were detectable in the urine before ischemia. Following ischemia there were dramatic increases in urinary free iron, increasing by 10- to 20-fold following ischemia and reperfusion (Table 2).

Nephron site of protection by iron chelators

GFR in the two control groups was similar (inulin: 220 ± 39 μl/min, dextran: 247 ± 63 μl/min; Fig. 4). Deferoxamine (50 mg/kg/hr) was again found to be protective of inulin clearance (490 ± 102; $P < 0.05$ vs. dextran or inulin). Inulin-conjugated deferoxamine was equally protective as free deferoxamine (560 ± 73 μl/min). On the other hand, dextran-conjugated deferox-

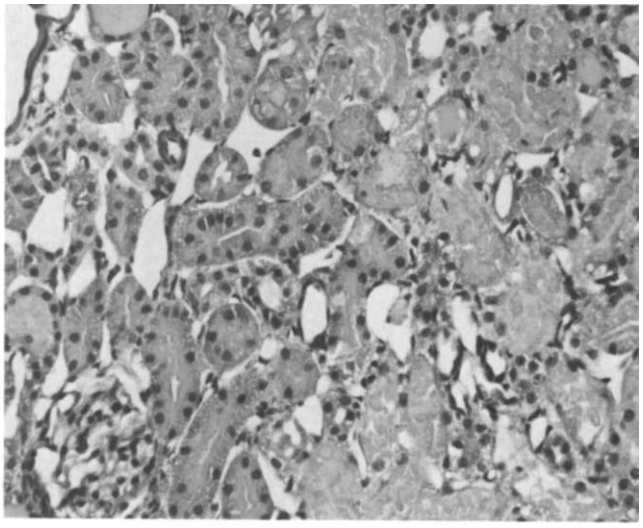

Fig. 2. *Photomicrograph of kidney from a control animal showin extensive cast formation and patchy necrosis.* Magnification ×300.

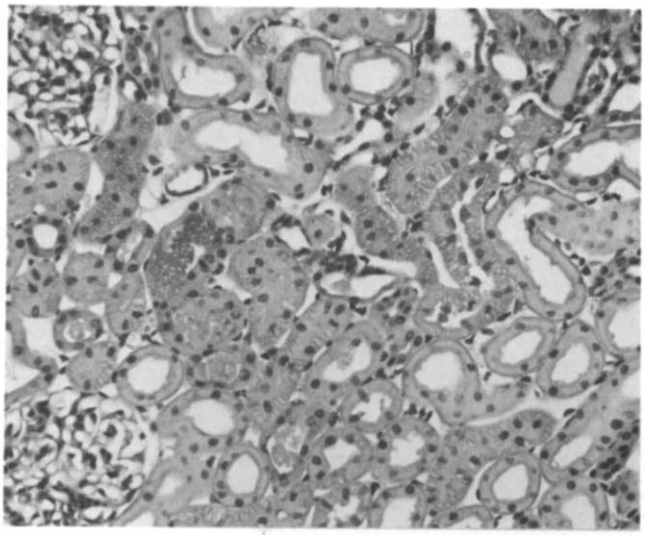

Fig. 3. *Photomicrograph of kidney from a deferoxamine-treated animal showing less extensive brush border loss, cast formation and cell necrosis than control kidney (Fig. 2).* Magnification ×300.

amine provided no protection (225 ± 60 μl/min, NS vs. dextran or inulin; $P < 0.05$ vs. deferoxamine) despite equivalent plasma deferoxamine levels. Plasma deferoxamine after infusion of 200 mg/kg/hr (a higher dose than that used to provide protection in this experiment) was 0.158 ± 0.02 mM in three rats sampled at three time points each. There was no significant variation of plasma deferoxamine over time. Plasma deferoxamine level in dextran-conjugated deferoxamine-infused rats ($N = 7$) was 0.130 ± 0.01 mM (not significantly different).

Discussion

Substantial data now exists to suggest that oxygen free radicals mediate injury following renal ischemia. Inhibitors of xanthine oxidase to prevent superoxide radical formation [2, 3, 5], superoxide dismutase to remove superoxide radical [2–5] and numerous scavengers of hydroxyl radical, such as dimethylthiourea, dimethylsulfoxide and glutathione [2, 23, 24], reduce renal injury following ischemia. Several of these radical scavengers have also been shown to limit postischemic lipid peroxidation [2, 3]. Although it is hypothesized that oxygen free radicals are produced only during the reperfusion of an ischemic organ, evidence for this relationship in vivo is limited. For example, lipid peroxidation does not appear to occur to any great extent during ischemia, but occurs promptly during reperfusion [2, 3, 25]. Administration of free radical scavengers only during the reperfusion period has occasionally been demonstrated to protect against renal injury [5], but in the majority of studies animals were given free radical scavengers before ischemia. In the present study all experimental manipulations were made at the conclusion of a 60 minute period of renal ischemia and, therefore, affected only reperfusion-associated events. The findings are consistent with an effect of free radicals to produce reperfusion-mediated, rather than ischemia-mediated, injury.

Because oxygen free radicals, including hydroxyl radical, produce postischemic renal injury a role of iron to mediate hydroxyl radical formation and/or lipid peroxidation would not be surprising. However, there is little data to support such a relationship in the kidney. In postischemic injury of the myocardium a role of iron has been suggested by preliminary studies [26]. Furthermore, iron has been demonstrated to participate in oxidant-mediated injury in other settings [14, 27–30]. We found a prominent role for iron in postischemic injury of the kidney. Administration of the iron chelator deferoxamine during the first hour of reperfusion resulted in improved renal function (GFR) and renal histologic findings and largely prevented postischemic lipid peroxidation. In previous studies of postis-

Table 2. Effect of ischemia and reperfusion on urinary free iron excretion

	Urinary free iron *pmol/30 min*
Baseline	
before ischemia	138 ± 54
Reperfusion	
15–45 min	1590 ± 776
45–75 min	1249 ± 506

Values are mean ± SE. $N = 8$ ($N = 6$ for second reperfusion period).
[a] $P < 0.05$ vs. baseline
[b] $P < 0.08$ vs. baseline

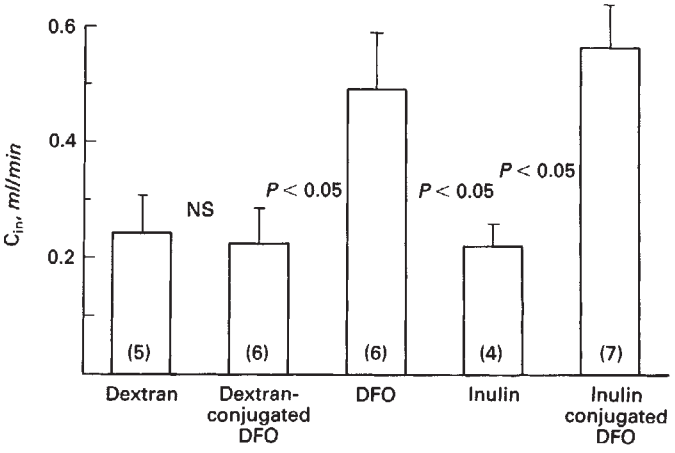

Fig. 4. *Effect of deferoxamine (DFO) compounds on GFR after renal ischemia.* Numbers in parentheses represent number of animals studied. NS, not significantly different.

chemic renal injury there was good correlation between the GFR at 24 hours and the amount of lipid peroxidation with the extent of histologic injury and renal tubular dysfunction [2, 23]. This beneficial effect of deferoxamine could not be attributed to an iron-independent effect since deferoxamine caused a decrease in blood pressure (potentially harmful rather than beneficial) and no effect on urine flow rate, GFR, renal blood flow, sodium or osmolar excretion. Also, iron-saturated deferoxamine had no protective effect. Conversely, provision of additional iron in the form of EDTA-$FeCl_3$ at the time of reperfusion exacerbated the postischemic decline in GFR and lipid peroxidation. We infused EDTA-$FeCl_3$ rather than $FeCl_3$ because free iron is rapidly bound by transferrin and other binding proteins in the plasma and would not be available to participate in free radical reactions [18]. EDTA-$FeCl_3$ circulates and is filtered by the kidney. The iron in this complex is bound loosely so that it can catalyze hydroxyl radical formation [16]. Therefore, during reperfusion iron exacerbated renal injury and iron chelation reduced renal injury.

The assay employed to measure lipid peroxidation, the thiobarbituric acid test, deserves further comment. Lipid peroxidation involves a number of complex reactions which include initiation steps by free radicals to form lipid hydroperoxides, propagation reactions, and ultimately termination steps [7, 31]. Many intermediate products are formed, and measurements of these intermediate products may give important information about these peroxidation reactions. In the assay for MDA lipid hydroperoxides which have been formed in vivo are decomposed by acid heating in vitro to yield thiobarbituric acid-reactive substances including MDA [32]. Both in vivo formation of lipid hydroperoxides and further decomposition in vitro are iron dependent [32]. Although MDA is formed in vivo, it is also metabolized and the amounts present in vivo are small [32]. Unfortunately, there is no wholly satisfactory method for measuring lipid peroxidation in vivo. In the present study we interpret the results to indicate that provision of iron as EDTA-$FeCl_3$ resulted in conditions more favorable for the formation of lipid hydroperoxides and subsequent degradation products. Conversely, deferoxamine produced conditions less favorable for formation of lipid hydroperoxides and their degradation products. It is also important to remember that although lipid peroxidation is strong evidence that free radicals have been present, tissue injury can also result from free radical reactions with proteins, carbohydrates, and DNA. Therefore, assessment of lipid peroxidation by any method is most useful to indicate that free radicals have been generated.

The source of the iron which was involved in postischemic injury and which was bound by deferoxamine is not clear. Several possibilities exist. Iron in tissues is predominately stored as ferritin in the form of ferric iron (Fe^{3+}). Reduction of the iron to ferrous iron (Fe^{2+}) results in its mobilization [6]. Release of iron from ferritin can be mediated by xanthine oxidase, primarily via superoxide radical [8–10]. Therefore, the postischemic production of superoxide radical could result in the release of free iron from ferritin. The ferritin could be either cytosolic or lysosomal in origin [33]. We were unable to demonstrate a postischemic increase in renal venous plasma free iron. Either no iron was released into the venous circulation or released iron was rapidly bound by proteins and could not be detected in our assay. On the other hand, we observed a striking increase in free iron in the urine following ischemia. Urine, which is for the most part protein-free, would be unable to bind iron released from renal tubular epithelial cells during ischemia or reperfusion. Therefore, iron released into the urinary space might be particularly dangerous in terms of promoting free radical formation and lipid peroxidation. This might explain the sensitivity of the kidney, in general, and of the brush border, in particular, to postischemic injury [34].

When deferoxamine which had access to the urinary space (free deferoxamine or inulin-conjugated deferoxamine) was provided during reperfusion protection was seen. Conversely, dextran-conjugated deferoxamine, which would undergo little filtration and thus not be present in the urinary space in a significant concentration during the first critical 20 to 40 minutes of reperfusion, provided no protection. These findings suggest that the free iron detectable in the urine was the critical iron pool in mediating postischemic renal dysfunction and lipid peroxidation. Because inulin-conjugated deferoxamine was as protective as free deferoxamine, substantial entry into cells would not seem necessary. However, since ischemia might render cells more permeable to larger molecules such as inulin, we cannot be certain that the only active site of deferoxamine was the extracellular space. Nevertheless, it is unlikely that cells with substantial permeability to inulin could retain intracellular electrolytes to remain viable. Because dextran-conjugated deferoxamine did not provide protection, a vascular site of free radical production (such as, endothelial cells) cannot be the primary site of iron-mediated postischemic injury.

Consistent with the hypothesis that free radicals are generated in or near the urinary space are previous findings that only those free radical scavengers which undergo glomerular filtration and/or tubular secretion (superoxide dismutase, dimethylthiourea, dimethylsulfoxide, glutathione) are protective [2, 23, 24]. Catalase, which is too large to be filtered, was not protective [2]. These findings do not require that all postischemic free radical formation occurs in the urinary space. Intracellular sources of postischemic O_2^- and H_2O_2 are cytoplasmic xanthine oxidase and the mitochondrial electron transport chain [1]. However, if iron which is released gains entry to the urinary space (as we have demonstrated) then the final step of $OH\cdot$ formation could take place there. Loss of xanthine oxidase into the urinary space could accentuate this process. Iron might also be loosely bound to the lipid membrane of the extensive brush border and thus direct injury to this location. Deferoxamine in the urinary space could bind this iron and inhibit its harmful effects.

Although the Haber-Weiss reaction was suggested above to be the manner whereby free ferrous iron mediates injury, other recent in vitro data suggests another possible important mechanism. Free iron, particularly when there is molar equivalence of Fe^{2+} and Fe^{3+}, can directly initiate lipid peroxidation [11, 12]. Proponents of this mechanism suggest that oxygen free radicals are most important in reducing Fe^{3+} to Fe^{2+} to attain the most favorable ratio of the two iron species [11, 12]. These phenomena are not readily examined in vivo in complex organs. The present study is consistent with either possibility and, regardless of the chemical reaction involved, clearly demonstrates a critical role of iron in mediating postischemic renal injury.

These observations have a number of important clinical implications. Deferoxamine has been safely used in man. Intravenous infusion of deferoxamine in doses comparable to those

used in the present study (30 to 50 mg/kg) have been routinely used for other indications [35, 36]. Therefore, deferoxamine might be effective in limiting reperfusion injury in man. Examples which immediately come to mind are revascularization procedures and organ transplantation where periods of ischemia are followed by controlled reperfusion. Iron may also be responsible for or contribute to hemoglobin- and myoglobin-induced acute renal failure. Hemoglobin can catalyze the Haber-Weiss reaction in vitro [37]. If iron from methemoglobin is released as free ferric iron, it too would be amenable to complexation by deferoxamine. In this setting deferoxamine would limit any additional iron-promoted injury. Studies from our laboratory suggest that this is indeed the case [38].

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