Oxygen Free Radical Induced Damage in Kidneys Subjected to Warm Ischemia and Reperfusion

*Protective Effect of Superoxide Dismutase**

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Superoxide anion free radical (O_2^-) has been implicated in the pathogenesis of tissue injury consequent to ischemia/reperfusion in several different organs, including heart and bowel. Superoxide dismutase (SOD), an enzyme free radical scavenger specific for O_2^-, has been used successfully to protect these organs from structural damage during reoxygenation of ischemic tissue. It has been suggested that the catalytic action of xanthine oxidase in injured tissue is an important source of O_2^- during reoxygenation. In order to evaluate the potential of SOD to protect against kidney damage resulting from transient ischemia followed by reperfusion with oxygenated blood, a model of warm renal ischemia was studied. LBNF1 rats underwent right nephrectomy and occlusion of the left renal artery for 45 minutes. Survival in the group of ischemic untreated rats (N = 30) was 56% at 7 days and serum creatinine was greatly elevated ($p < 0.01$) in rats remaining alive over the full 7-day period. In strong contrast to these results, all of the animals treated with SOD before reperfusion (N = 18) were alive after 7 days similar to sham operated control rats (N = 8). Serum creatinine in the SOD treated rats was significantly elevated only to postoperative day 3 and thereafter returned to normal. Rats treated with inactive SOD (N = 4) or SOD before ischemia (N = 4) had decreased survival rates compared to ischemic untreated animals and prolonged elevation of serum creatinine. When the ischemia time was extended to 60 minutes, only 19% of the untreated animals (N = 16) survived at 7 days whereas nearly 60% of the SOD-treated animals survived (N = 19). Serum creatinine was greatly elevated during the full 7-day observation period in all surviving rats in the untreated ischemic group, whereas serum creatinine returned to normal ($p < 0.05$) after 4 days in the surviving rats treated with SOD. To test whether the action of xanthine oxidase contributed to the kidney damage after reoxygenation, 45 min. ischemic rat kidneys were treated with allopurinol. All of the animals treated with allopurinol (N = 12) were alive at 7 days. Serum creatinine values returned to normal after the episode of ischemia and reperfusion but more slowly than after SOD treatment. Histologic evaluation of kidney tissue taken from animals after ischemia alone showed extensive renal tubular damage, which was essentially absent in kidneys from SOD-treated animals. Allopurinol prevented the extensive injury to renal tubules after ischemia and reperfusion but was not nearly as effective as SOD. Our findings support the proposal that O_2^- plays a critical role in the structural and functional damage to kidneys that results from reoxygenation of ischemic tissue and further that the oxygen radicals are in part derived from the action of xanthine oxidase. When administered immediately before reoxygenation, SOD effectively prevents postischemic kidney injury.

WARM RENAL ISCHEMIA is an inevitable consequence of a number of common clinical situations and operative procedures. For example, cardiac arrest with recovery, heminephrectomy, or other types of renal vascular procedures are frequently associated with transient warm renal ischemia. Ischemic renal damage is a serious and unsolved problem in the subsequent fate of kidneys removed for renal transplantation. The acute tubular necrosis that develops as a direct result of renal ischemia significantly increases the posttransplant morbidity and the early loss of transplanted kidneys.[1] Acute tubular necrosis after renal transplantation is frequent, occurring in

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30–60% of the recipients of cadaver donor kidneys and up to 10% of the recipients of living, related-donor kidneys.[2–4] Successful restoration of renal function in patients whose own or donated kidneys have been subjected to temporary warm renal ischemia could be confidently expected rather than hoped for, as is the current situation if kidney injury as a result of transient ischemia could be reduced or eliminated.

It has been previously observed that reperfusion of ischemic myocardium or kidneys produces much more damage than that caused by the period of ischemia alone.[5,6] The role of superoxide radicals in the process of ischemia-reperfusion induced tissue damage has recently been investigated in a number of organ systems. These studies suggest that although ischemia potentiates tissue damage, it may actually be the reentry of oxygenated blood into the ischemic organ during reflow that initiates tissue damage. Oxygen is normally metabolized completely to water with only low quantities of the reactive intermediate products of oxygen metabolism formed. Transient ischemia and subsequent reperfusion appears to disrupt the normally tightly controlled metabolism of oxygen, thus enhancing the rate of production of reactive oxygen species such as superoxide anion free radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl free radical ($HO^\cdot$).

Reactive oxygen species have been implicated as mediators of tissue damage during organ reperfusion in several experimental models, including the globally ischemic myocardium,[7] ischemia/reperfusion-induced increased capillary permeability in the small intestine and lung,[8,9,11] ischemic tissue injury in island skin flaps,[10] and in a variety of other systems.[12,13]

Superoxide dismutase (SOD), which catalytically dismutes two molecules of O_2^- specifically and rapidly to O_2 and H_2O_2 acts in biological systems as an antioxidant.[14] When administered during or immediately after the initiation of certain types of tissue injury, SOD has proved effective in substantially diminishing the extent of injury.[15]

Oxygen free radicals or reactive species can disrupt the integrity of the endothelium, which lines the vascular system.[16] Loss of endothelial integrity with the consequent extravasation of intravascular fluid and blood cells into the previously ischemic area could be the triggering event in reperfusion injury. Several molecular mechanisms have been proposed to explain this process of oxygen radical cytotoxicity. Experimental evidence suggests that in several models of ischemia-reperfusion injury, the enzyme xanthine oxidase is the source of damaging amounts of oxygen radicals.[17] This has been shown indirectly with the demonstration of protection by the xanthine oxidase inhibitor allopurinol. Accumulation of both xanthine oxidase and its substrates hypoxanthine and xanthine during ischemic episodes has been described to poise the system to tissue injury on reoxygenation.

The purpose of this study was to determine the extent to which superoxide anion radicals are a contributing factor in the pathogenesis of renal damage from transient warm renal ischemia and to assess the effectiveness of SOD as a protective agent as evaluated by alterations in mortality of the experimental animals and by assessment of function and histopathology of the affected kidneys. The role of tissue xanthine oxidase as a source of oxygen radicals was investigated by the use of the xanthine oxidase inhibitor, allopurinol.

Materials and Methods

Surgical Procedure

Male LBNF1 rats (200–250 g), housed four to a cage, fed a pellet diet, and given tap water to drink were anesthetized with intraperitoneal injections of 3.6% chloral hydrate (1 ml/100 g). Following induction of general anesthesia, a midline abdominal incision was made. The left renal vascular pedicle was isolated in close proximity to the aorta. A 0.5 ml sample of blood was obtained from the inferior vena cava before the intravenous systemic administration of heparin, 0.4 ml (80 I.U.). The left renal vascular pedicle was occluded with an atraumatic vascular clip for either 45 or 60 minutes. To minimize fluid loss through evaporation and tissue dehydration, all exposed tissues were moistened with Ringer's lactate solution after which the abdominal incision was temporarily closed with clamps. Body temperature of the rats was maintained during surgery with a thermistor-controlled infrared lamp. Rodent core body temperature was maintained at 36–37 C by using a rectal probe and a Thermistemp Temperature Controller, Model 71A (Yellow Springs Instrument Co., Yellow Springs, Ohio).

A right nephrectomy was performed several minutes before the end of the ischemic period for the contralateral kidney. At this point, the heparinized animal also received intravenous Ringer's lactate solution to replace intravascular volume lost as a result of the preoperative blood sample and oozing from the wound edges. Where indicated, either SOD, allopurinol, or the control solution of 5% dextrose in water was administered intravenously 2 minutes before reperfusion of the remaining ischemic left kidney. The left kidney was observed for 4 minutes after unclamping, and, if visible evidence of restored blood flow was not obtained within 2 minutes or less, the animal was not included in the study. The inability to restore blood flow in these cases was attributed to inadequate systemic anticoagulation and subsequent vascular thrombosis. He-

mostasis of the wound edges was attained with a battery-operated cautery. Ringer's lactate solution (2 ml/100 g) was placed in the peritoneal cavity at the conclusion of the operative procedure. The abdominal wall was then closed in two layers with permanent suture, and the animal was allowed to recover in warm surroundings. Animals were observed for a maximum of 7 days and were killed at indicated times for histologic examination of their previously ischemic left kidneys.

Experimental Protocols

Eleven different groups of experimental animals were prepared. All rats underwent right renal nephrectomy; thus, all the experimental groups were dependent on the single left kidney for renal function. These groups were: A. Sham-operated animals, which underwent right nephrectomy only. B. Untreated ischemic rats, which underwent left renal ischemia by occlusion of the renal artery for 45 minutes before blood flow was restored. Immediately before reflow, a solution of 5% dextrose in water was injected intravenously. C. SOD-treated ischemic rats, which underwent the same operative procedure as group B but were treated with SOD (6.5 mg/kg) as a bolus injection intravenously immediately before renal reperfusion. D. SOD-treated preischemic animals, which underwent the same operative procedure as group B but were treated intravenously with SOD (6.5 mg/kg) 2 minutes before the 45 minute period of temporary renal artery occlusion. No additional enzyme was injected before reperfusion. E. Inactivated SOD-treated ischemic animals, which underwent the same operative procedure as group B but were treated with heat inactivated SOD (6.5 mg/kg) immediately before renal perfusion after 45 minutes of ischemia. F. Low-dose SOD-treated ischemic rats, which underwent the same operative procedure as group B but were treated with 3.25 mg/kg SOD in a bolus injection immediately before renal reperfusion. G. High-dose, SOD-treated ischemic rats, which underwent the same operative procedure as group B but were treated with 13 mg/kg SOD as a bolus injection immediately before reperfusion. H. Nonspecific protein treated ischemic rats, which underwent the same operative procedure as group B but were given albumin (6.5 mg/kg) as a bolus injection immediately before renal reperfusion. I. Allopurinol-treated ischemic rats, which underwent the same operative procedure as group B but were treated with allopurinol (100 mg/kg) injected as a bolus immediately before renal reperfusion. J. Extended ischemic rats, which underwent occlusion of the renal artery for 60 minutes before blood flow was restored. K. SOD-treated extended ischemic rats, which underwent the same operative procedure as group J but were treated with SOD (6.5 mg/kg) as a bolus injection immediately before renal reperfusion (See Table 1 for the complete listing of the experimental groups).

Following the operative procedure and subsequent recovery period, the experimental animals were allowed to return to their cages. A pellet diet and water was available *ad lib.* A blood sample (0.2–0.3 ml) was obtained from the rat tail daily for 7 days and used for determination of the serum creatinine concentration.

Superoxide dismutase (SOD). SOD, diluted appropriately in 5% dextrose in water, was administered intravenously in variable quantities but a uniform volume (3.25, 6.5, and 13 mg/kg) 2 minutes before release of the left renal arterial clamp. The enzyme was diluted in this manner to administer a constant volume intravenously to all animals despite the variation in total dose of the enzyme. SOD is known to be rapidly cleared by the kidneys[29]; therefore, the right nephrectomy, which was performed on all animals, and the bolus injection of the SOD just before reperfusion ensured maximum exposure of the enzyme to the injured tissue. Heat inactivated SOD was prepared by autoclaving a solution of SOD at 110 C for 1 hour autoclave at 110 C for 1 hour. No enzymatic activity was detectable after this treatment when the preparation was assessed by the standard method.[34]

Allopurinol. Allopurinol (kindly donated by Burroughs Wellcome Co., Tuckahoe, N.Y.) was administered intravenously as the sodium salt prepared by the addition of 1 molecular equivalent of sodium hydroxide; that is, 137 mg allopurinol required 1 ml of 1 N NaOH. A final concentration of 25 mg allopurinol/ml was obtained by dilution with 5% dextrose in water at 37 C.

Albumin. Albumin (Sigma Chemical Co., St. Louis, Missouri) was administered intravenously as a nonenzymatically active protein control. Albumin was diluted to a final concentration of 2 mg/ml with 5% dextrose in water.

Tissue preparation for histology. Animals were killed at either 3 days or 7 days after operation, and the remaining left kidney was removed for histologic examination. After rapid total excision, each kidney was immediately placed in 4% formaldehyde with 2% sodium acetate. The tissues were then embedded in paraffin, cut to a thickness of 6 micrometers, and stained with hematoxylin and eosin.

Morphologic evaluation. Coded histopathologic sections were examined for morphological changes produced by transient warm ischemia. This evaluation was made by an observer who had no knowledge of the treatment group from which the specimens came. Transient warm ischemia resulted in necrosis of the proximal convoluted tu-

bule. Glomeruli appeared to be normal. Ischemic necrosis was graded from 1 to 4 on the third and seventh days after renal ischemia according to the criteria outlined by Jablonski.[47] (See Table 3.)

Creatinine. Serum creatinine, expressed as mg/dl (mg%), was determined by its reaction with sodium picrate in alkaline solution.[30] The resultant creatinine picrate complex was measured spectrophotometrically. Reagents were obtained as a kit: Ultra Chem Creatinine 64972 (HarleCo, Division of EM Industries, Gibbstown, NJ).

Statistical analysis of data. The individual creatinine determination for a single day on a single animal was considered the unit of observation. Within each group of animals, a mean creatinine concentration ± standard error of the mean was calculated for each day. Since the sample size was adequate, a one-way analysis of variance was used to determine whether the daily mean creatinine differences between the treated and control groups were significant. The probability of significant differences in survival between treated and control groups was analyzed by Fishers exact probability determination method.

TABLE 1. *Per Cent Survival of Rats Following Transient Renal Ischemia and Treatment with Superoxide Dismutase and Allopurinol*

Group*		N	Survival on Day 7 (%)
	Warm Renal Ischemia of 45 Minutes		
A	Untreated control: sham operated	8	100
B	Ischemia untreated	30	56
C	Ischemia-SOD (6.5 mg/kg)	18	100†
D	Pre-ischemia SOD (6.5 mg/kg)	4	25
E	Ischemia-inactivated SOD (6.5 mg/kg)	4	25
F	Ischemia-SOD (3.25 mg/kg)	4	50
G	Ischemia-SOD (13 mg/kg)	4	100
H	Ischemia-albumin (6.5 mg/kg)	4	50
I	Ischemia-allopurinol (100 mg/kg)	12	100†
	Warm Renal Ischemia of 60 Minutes		
J	Ischemia untreated	16	19
K	Ischemia-SOD (6.5 mg/kg)	19	58‡

* Rats in all groups underwent right nephrectomy.
† $p < 0.01$ and ‡ $p < 0.05$ compared with untreated ischemia (Group B or J).

Results

The survival of animals with acute renal failure under all experimental conditions was assessed over a 7-day postischemic period (Table 1). At 7 days, only 56% of the rats that underwent 45 minutes of renal artery occlusion before reflow but with no treatment remained alive. In contrast, all the SOD-treated rats were alive after 7 days when the enzyme was administered immediately before reperfusion following 45 minutes of renal ischemia ($p < 0.01$). SOD-treatment significantly improved the survival of animals undergoing 60 minutes ($p < 0.01$) of warm renal ischemia. After a 60-minute ischemic period followed by a preflow injection of dextrose in water, only 19% of the animals remained alive, whereas with SOD treatment 58% of the animals were alive at postoperative day 7. When rats were given the same concentration of SOD before 45 minutes of ischemia rather than immediately before reflow, the 7-day survival rate of these animals was even less than for ischemic nontreated animals. Animals treated with albumin or heat-inactivated SOD following the 45-minute period of renal ischemia showed a percentage survival at 7 days that was the same or less than the ischemic nontreated animals, respectively. When SOD was administered at a higher concentration (13 mg/kg), no perfusion-related mortality was observed similar to the results with administration of 6.5 mg/kg SOD (Table 1). When SOD was administered at one-half the initial concentration (3.25 mg/kg) immediately before reperfusion, there was no improvement in survival compared with the ischemic nontreated animals. This group did exhibit a significant reduction in serum creatinine ($p < 0.05$) on the first and third days after ischemia, however, indicating at least some benefit to the surviving animals from this lower concentration of SOD at reperfusion. The rates of survival throughout the 7-day period of observation for selected experimental groups show that the maximum rate of mortality occurred between postoperative days 1 and 4 (Figs. 1 and 2).

Samples of serum were analyzed for creatinine concentration in all experimental groups immediately before operation (day 0) and for 7 postoperative days (Table 2). Serum creatinine concentrations of the sham-operated group obtained over the 7-day observation period reflected normal serum creatinine values. The serum creatinine concentration was significantly higher in the surviving animals that underwent 45 minutes of ischemia with no treatment compared with the sham-operated control group on postoperative days 1 through 6. In strong contrast, however, animals experiencing 45 minutes of renal ischemia but treated with 6.5 mg/kg SOD immediately before reflow showed a rapid return to normal serum creatinine values in all animals after a transient rise on postoperative days 1, 2, and 3.

When the period of warm renal ischemia was extended to 60 minutes, serum creatinine concentrations of the few surviving ischemic nontreated rats were greatly elevated throughout the entire 7-day postoperative period com-

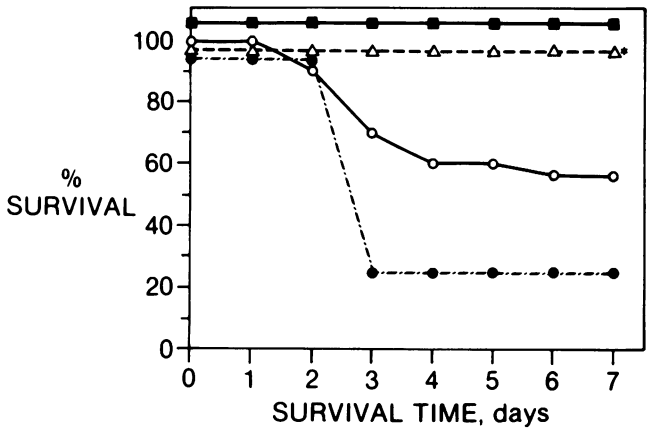

FIG. 1. Per cent survival over a 7-day postoperative period of rats that have undergone 45 minutes of renal ischemia and treatment with superoxide dismutase (SOD). Rats in all experimental groups had the right kidney removed. Untreated control (sham-operated) rats with right nephrectomy only (N = 8) (■ — ■); left renal artery occluded for 45 minutes and 5% dextrose in water injected intravenously 2 minutes before reflow (N = 30) (○ — ○); left renal artery occluded for 45 minutes and 6.5 mg/kg of SOD injected intravenously 2 minutes before reflow (N = 18) (△ — △); 6.5 mg/kg SOD injected intravenously 90 seconds prior to ischemic clamping for 45 minutes followed by reflow (N = 4) (● — ●).

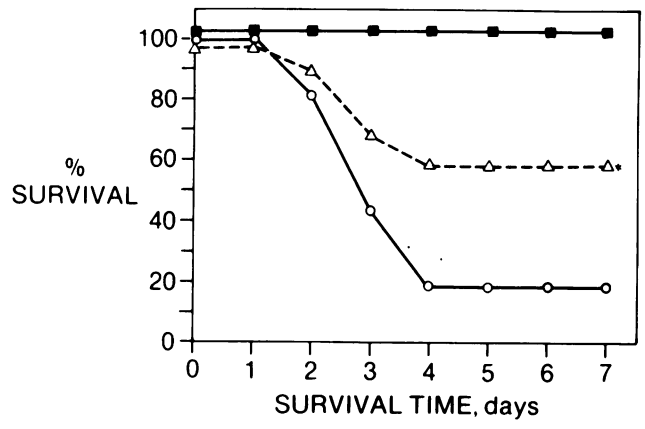

FIG. 2. Per cent survival over a 7-day postoperative period of rats that have undergone 60 minutes of renal ischemia and treatment with superoxide dismutase (SOD). Rats in all experimental groups had the right kidney removed. Untreated controls (sham-operated) rats with right nephrectomy only (N = 8) (■ — ■); left renal artery occluded for 60 minutes and 5% dextrose in water injected intravenously 2 minutes before reflow (N = 16) (○ — ○); left renal artery occluded for 60 minutes and 6.5 mg/kg of SOD injected intravenously 2 minutes before reflow (N = 19) (△ — △).

pared with normal values ($p < 0.01$). Although SOD did not prevent death in all cases after 60 minutes of warm renal ischemia, enzyme treatment significantly restored renal function in the surviving rats after 3 postoperative days (Table 2). At day 3, the peak evaluation of serum creatinine of the untreated 60 minute ischemic rats was seen. Treatment with SOD, therefore, promoted an earlier

TABLE 2. *Serum Creatinine Levels in Rats Subjected to Transient Renal Artery Occlusion and Various Treatments*

	Mean Serum Creatinine ± SEM (mg %)‖							
Postoperative Day	0	1	2	3	4	5	6	7
Untreated control: sham operated	1.61 ± 0.10	2.25 ± 0.31	2.53 ± 0.35	2.07 ± 0.17	2.01 ± 0.10	1.81 ± 0.12	1.55 ± 0.13	1.51 ± 0.16
Ischemia: 45 Minutes								
Ischemia untreated	1.89 ± 0.11	5.74 ± 0.39*	7.06 ± 0.55*	5.67 ± 9.59*	4.19 ± 0.54†	3.36 ± 0.52†	2.68 ± 0.25	2.14 ± 0.17
Ischemia-SOD (6.5 mg/kg)	1.74 ± 0.08	4.50 ± 0.19‡	4.72 ± 0.49‡	3.66 ± 0.45‡	3.09 ± 0.54	2.61 ± 0.37	2.33 ± 0.23	2.15 ± 0.16
Pre-ischemia SOD (6.5 mg/kg)#	1.90 ± 0.12	5.62 ± 0.24	8.30 ± 0.20	9.00 ± 0.00	9.20 ± 0.00	5.20 ± 0.00	4.20 ± 0.00	3.30 ± 0.00
Inactivated SOD (6.5 mg/kg)#	1.72 ± 0.10	5.30 ± 0.28	7.07 ± 0.34	8.67 ± 0.42	5.15 ± 0.00	4.82 ± 0.00	3.71 ± 0.00	3.41 ± 0.00
Ischemia-SOD (3.25 mg/kg)#	1.55 ± 0.22	3.57 ± 0.80§	5.47 ± 1.92	2.00 ± 0.50§	2.10 ± 0.30	1.90 ± 0.40	1.60 ± 0.40	1.45 ± 0.25
Ischemia-allopurinol	1.97 ± 0.13	4.80 ± 0.33	5.29 ± 0.52§	4.69 ± 0.60	3.81 ± 0.33	3.64 ± 0.26	3.24 ± 0.25	3.06 ± 0.24
Ischemia: 60 Minutes								
Ischemia untreated	1.95 ± 0.12	5.28 ± 0.40*	7.40 ± 0.44*	7.71 ± 1.16*	5.56 ± 1.81	5.26 ± 1.96*	4.46 ± 1.99*	3.90 ± 1.74*
Ischemia-SOD (6.5 mg/kg)	1.83 ± 0.12	4.72 ± 0.18	6.55 ± 0.46	5.49 ± 0.82§	4.46 ± 0.82	3.60 ± 0.69	2.70 ± 0.44§	2.22 ± 0.28§

* $p < 0.01$ compared to sham.
† $p < 0.05$ compared to sham.
‡ $p < 0.01$ compared to ischemia untreated.
§ $p < 0.05$ compared to ischemia untreated.
‖ N = 8 in all groups except where indicated.
N = 4.

return to normal renal function ($p < 0.05$). By postoperative days 6 and 7, serum creatinine levels had returned to normal in rats subjected to prolonged ischemia and treated with SOD.

It is important to note that SOD was effective in eliminating or reducing mortality and in restoring the serum creatinine concentration to normal values after the experience of warm renal ischemia only when the enzyme was administered immediately before reperfusion. SOD administered before left renal artery occlusion and contralateral nephrectomy (preischemia SOD) in fact enhanced mortality (Fig. 1 and Table 1) and did not prevent prolonged elevation of serum creatinine (Table 2). Similarly, heat-inactivated SOD failed to reduce serum creatinine following temporary renal ischemia (Table 2) in addition to enhancing the mortality (Table 1). The mechanism of enhanced mortality by these treatments is unknown but may result from the action of denatured enzyme. The lack of protective action of albumin eliminated the possibility that protection was the result of nonspecific protein effects. The protective effect provided by SOD, which occurred only when this enzyme was administered immediately before reflow, seems to establish firmly the pathological role of the superoxide anion free radical (O_2^-) in this model of temporary warm renal ischemia followed by reperfusion with well-oxygenated blood.

The ischemic and reperfused left kidneys were removed from experimental animals on days 3 and 7 and examined histologically for acute tubular damage. Animals used for histologic studies on day 3 were not included in the mortality data. Kidney damage was ranked by a pathologist who had no knowledge of the experimental treatments. The grading method used is described in Table 3. A summary of the histopathologic analysis of the treated and untreated rat kidneys showed that much less structural damage occurred in all groups of animals that were treated with SOD immediately before reflow (Table 4). Normal renal tubules are seen in the sham-operated animals (Fig. 3).

TABLE 3. *Grading Categories for the Histopathologic Evaluation of Rat Kidneys*

Grade	Description
0	Normal (Fig. 3)
1	Mitoses and necrosis of individual cells (Fig. 7)
2	Necrosis of cells in adjacent proximal convoluted tubules, with survival of surrounding tubules (Fig. 6)
3	Necrosis confined to the distal third of the proximal convoluted tubule with a band of necrosis extending across the inner cortex (Fig. 5)
4	Necrosis affecting all three segments of the proximal convoluted tubule (Fig. 4)

(Adapted from Jablonski P, Howden BU, Rae DA. An experimental model for assessment of renal recovery from warm ischemia. Transplantation 1983; 35:198–204.)

A period of untreated warm renal ischemia for 45 or 60 minutes produced a zone of tubular necrosis across the inner cortex of the rat kidney (Figs. 4 and 5). The most severe damage evaluated at Grade 4 was observed in the kidneys of rats subjected to 60 minutes of ischemia with no treatment (Fig. 4). The kidneys from rats subjected to 45 minutes of renal ischemia alone showed Grades 2 and 3 damage on day 3 (Fig. 5). Grade 2 tubular damage remained in the surviving animals on postischemia day 7 (Fig. 6).

In sharp contrast to the findings in the untreated isch-

TABLE 4. *Histopathology of Rat Kidney after Transient Renal Ischemia and Reperfusion with and without Treatment*

	Postoperative Day 3						Postoperative Day 7					
Histopathologic Grade	0	1	2	3	4	N	0	1	2	3	4	N
Untreated control: Sham operated	1	0	0	0	0	1	1	0	0	0	0	1
Ischemia of 45 Minutes:												
Ischemia untreated	0	0	1	2	0	3	0	1	2	0	0	3
Ischemia-SOD (6.5 mg/kg)	0	2	1	1	0	4	2	3	1	0	0	6
Ischemia-SOD (13 mg/kg)	—	—	—	—	—	—	0	4	0	0	0	4
Ischemia-allopurinol (100 mg/kg)	0	0	1	0	0	1	1	4	2	0	0	7
Ischemia-albumin (6.5 mg/kg)	0	0	0	1	0	1	0	0	0	1	0	1
Ischemia-inactivated SOD (6.5 mg/kg)	0	0	0	1	0	1	0	0	0	1	0	1
Ischemia of 60 Minutes:												
Ischemia only	0	0	0	0	1	1	0	0	0	1	0	1
Ischemia-SOD (6.5 mg/kg)	0	0	0	1	0	1	0	5	0	0	0	5

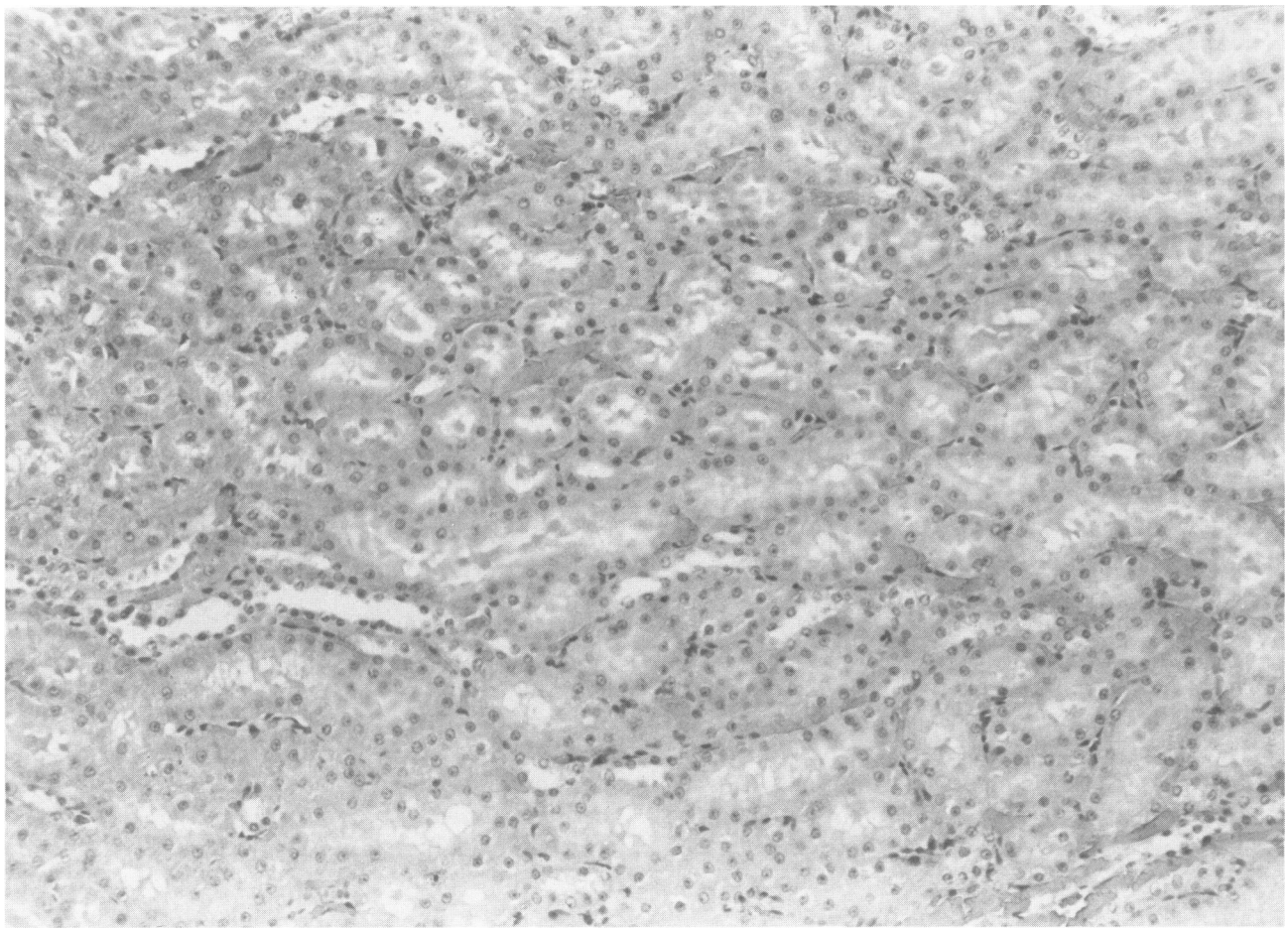

FIG. 3. Section of rat renal cortex taken on the third postoperative day from the left kidney of a sham-operated control rat and stained with hematoxylin and eosin. Renal tubules are normal. Representative of histopathologic Grade 0. Original magnification ×100.

emia group, the experimental group that received SOD immediately before reperfusion exhibited striking protection against acute tubular damage. The treated group tended to display only Grade 1 (Fig. 7) or Grade 2 tubular damage on postischemia day 3 and Grade 1 or the complete resolution of acute tubular damage by postischemia day 7 (Fig. 3).

The severe tubular damage that occurred in kidneys exposed to 60 minutes of warm renal ischemia, that is, Grade 4 and Grade 3 tubular damage on day 3 and day 7, respectively, was prevented when SOD (6.5 mg/kg) was administered immediately before kidney reperfusion following the 60-minute period of renal ischemia. Renal tubular damage was much less extensive in the treated animals and showed Grade 3 and Grade 1 tubular damage on day 3 and day 7, respectively.

Allopurinol, a competitive inhibitor of the enzyme xanthine oxidase, was tested in a manner similar to that employed with the SOD. When compared with untreated animals, all animals treated with allopurinol immediately before reperfusion survived the episode of 45 minutes of renal ischemia and reflow (Fig. 8). Treatment with allopurinol also resulted in significant restoration of serum creatinine to lower normal levels ($p < 0.05$) on postoperative day 4 (Table 2). The degree of renal tubular damage on postoperative day 7 in animals treated with allopurinol immediately before reperfusion appeared to be somewhat more extensive (Grades 1 and 2) than that seen in animals undergoing an identical 45 minute period of renal ischemia and treated with SOD (Grades 0 and 1). These experimental observations seem to indicate that although allopurinol prevents death from renal ischemia in this model and significantly reduces this structural damage of the reperfused kidney, it is not as effective a treatment as SOD for renal ischemia-reperfusion injury.

Discussion

These studies demonstrate that the administration of SOD, an oxygen radical scavenger specific for the detox-

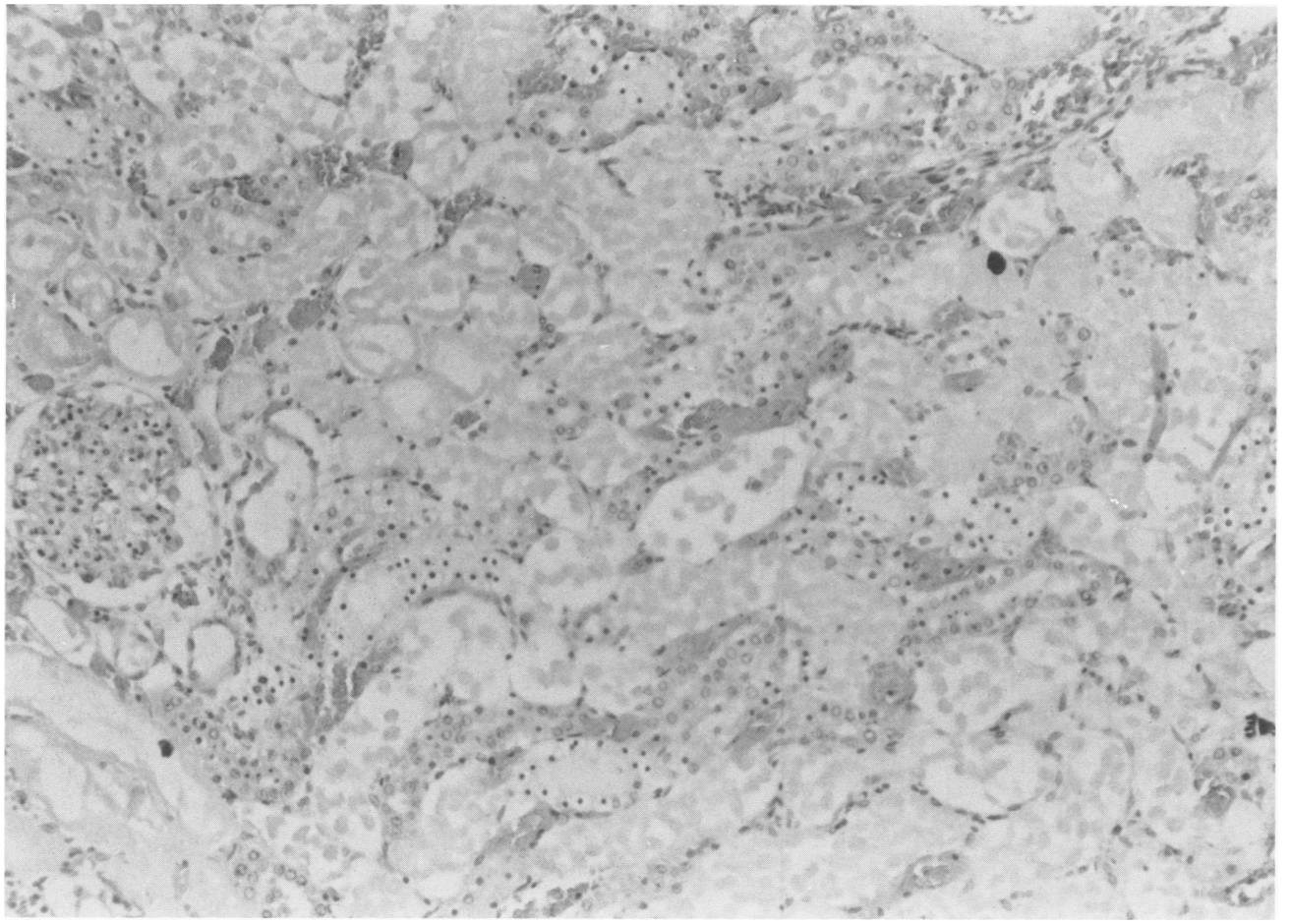

FIG. 4. Section of rat renal cortex taken on postoperative day 3 from the left kidney of an ischemic 60 minute untreated rat. Necrosis is seen in all parts of the proximal convoluted tubule. Representative of histopathologic Grade 4. Original magnification ×100.

ification of superoxide oxide anion (O_2^-), provided substantial protection against postischemic renal dysfunction. Without treatment at the time of reflow of oxygenated blood into the kidney, nearly 50% of animals died by the seventh postoperative day after 45 minutes of renal ischemia and 80% died at day 7 after 60 minutes of renal ischemia. In all groups treated with SOD during kidney reperfusion, survival following temporary renal ischemia and reflow was either complete after 45 minutes of warm renal ischemia or significantly improved after 60 minutes of ischemia. An important observation from these studies was that SOD administered before the period of ischemia did not prevent reperfusion-related mortality. Indeed the survival rate appeared to decline compared with the reperfused, untreated group. This suggests that an adequate concentration of active SOD must be present only in the initial reperfusion blood flow in order to be effective. The pathologic elevation of serum creatinine in rats subjected to 45 minutes and 60 minutes of ischemia was significantly diminished with SOD. Although the reduction of elevated serum creatinine by SOD treatment was more profound after the shorter period of ischemia, the data indicate that the enzyme can be effective against the severe injury caused by more lengthy ischemic periods in the kidney. Treatment with allopurinol, which prevents oxygen radical generation by inhibition of xanthine oxidase, also resulted in 100% survival after 45 minutes of temporary ischemia. Allopurinol produced a significant reduction in postischemia-induced elevation serum creatinine, as well. The ameliorative effect of allopurinol on creatinine levels was less than when SOD was administered after 45 minutes of renal ischemia, however. These experimental findings support a role for oxygen free radicals in the pathogenesis of postischemic renal dysfunction. The results also strongly suggest that xanthine oxidase contributes to the generation of oxygen free radicals during renal reperfusion.

Acute renal failure that is not associated with a primary glomerular or vascular disorder has been referred to as acute tubular necrosis. The end result of acute renal failure

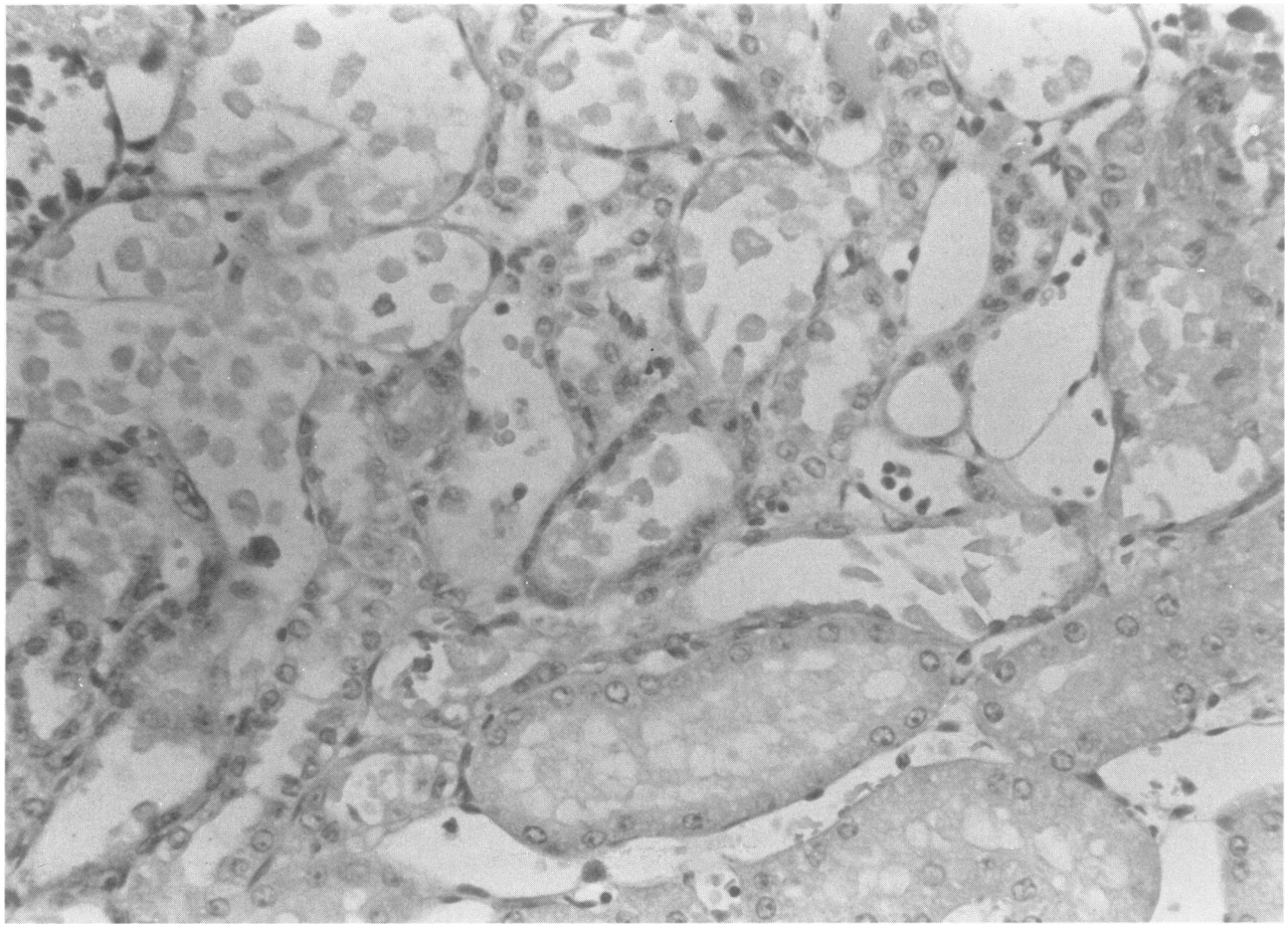

FIG. 5. Section of rat renal cortex taken on postoperative day 3 from the left kidney on an ischemic 45 minute untreated rat. Necrosis is seen in the distal third of the proximal convoluted tubules. Representative of histopathologic Grade 3. Original magnification ×200.

is a marked reduction in glomerular filtration rate. It has been proposed that in some manner vascular events within the kidney might lead to the complete cessation or profound diminution of glomerular filtration and thus precipitate acute renal failure.[18] Early studies of the rat kidney demonstrated that swelling of the glomerular capillary endothelial cells was a consistent consequence of obstruction of the rat renal artery for 60–120 minutes.[19] This endothelial cell swelling was accompanied by a decrease in renal blood flow that persisted even after reperfusion of the rat kidney. The decrease in renal blood flow that occurs after temporary ischemia was improved by treatment with SOD.[20,22] Alteration of renal blood flow may not be the primary factor responsible for improved glomerular filtration rate after temporary ischemia because there is usually a poor correlation between glomerular filtration rate and renal blood flow following ischemia-induced acute renal failure.

Oxygen free radicals have been implicated in the pathogenesis of cellular damage associated with oxygen toxicity and phagocyte-mediated inflammation.[23–25] In addition, superoxide anion radicals have been implicated as mediators in the increased capillary permeability induced by temporary ischemia in the small intestine and the lung,[8,9] brain damage resulting from cerebral ischemia,[21] the ischemia-induced necrosis observed in island skin flaps,[10] and finally as mediators in the morphologic disruption of small bowel epithelium and intestine noted to occur following temporary ischemia and reperfusion.[26,27,49] Superoxide anion radicals have previously been shown to play a role in the pathogenesis of ischemia-induced renal dysfunction.[20,22,28] Our work confirms these findings and, importantly, demonstrates that superoxide radicals are generated immediately after the onset of reperfusion. Unlike many of the previous studies, in our study both SOD and allopurinol were administered immediately before reflow; therefore, to be effective it was not necessary for these agents to be present within the

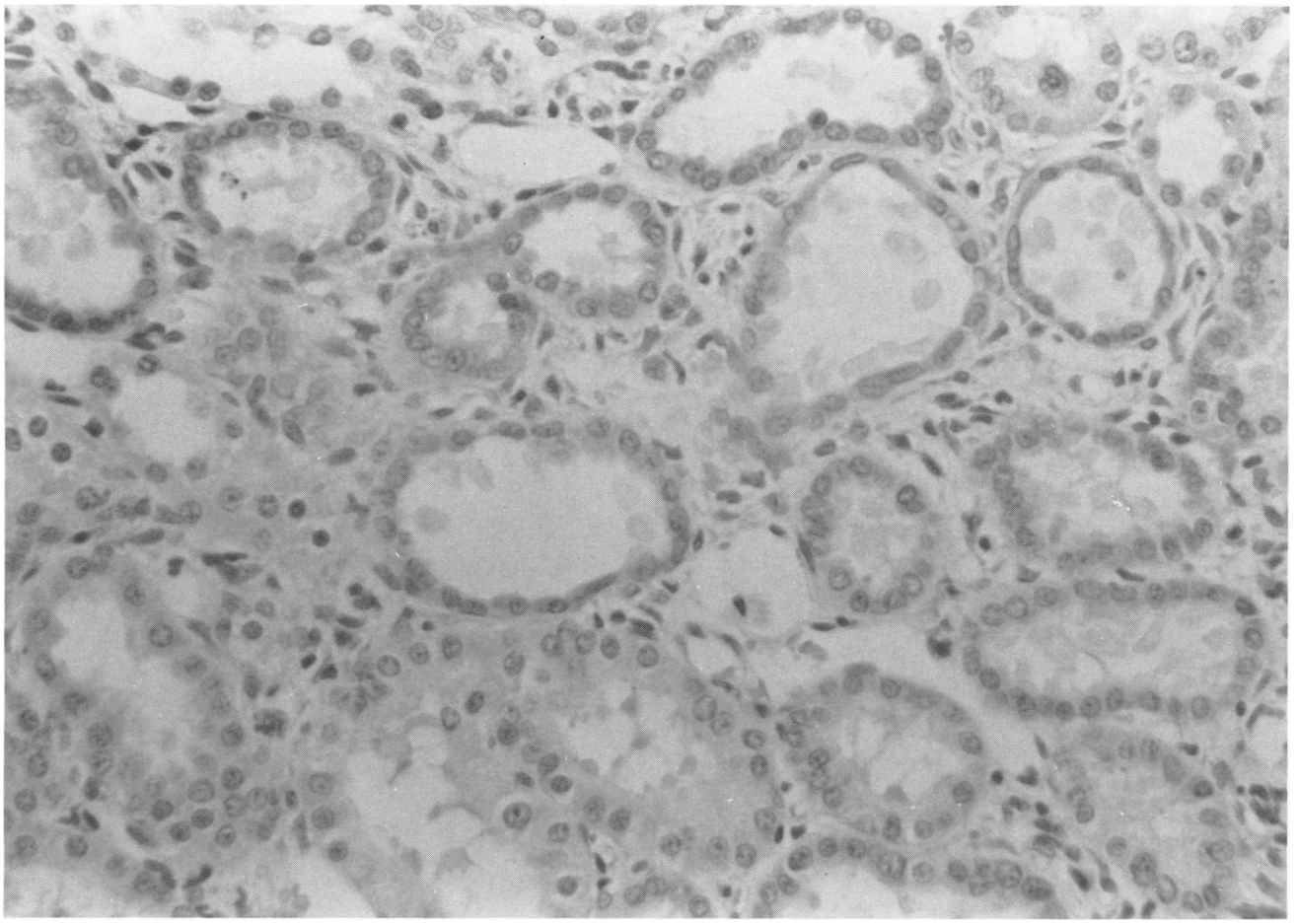

FIG. 6. Section of rat renal cortex taken on postoperative day 7 from the left kidney of an ischemic 45 minute untreated rat. Necrosis of a group of proximal convoluted tubules is seen. Representative of histopathologic Grade 2. Original magnification ×200.

kidney during the period of temporary ischemia. Only a single dose of either SOD or allopurinol prevented post-ischemia-related mortality when given immediately before reflow. Diminished survival, in fact, was observed when SOD was administered before the period of ischemia. These findings suggest that not only are superoxide anion radicals generated immediately upon the onset of reperfusion but also their pathological actions are initiated at this time. SOD is known to be cleared rapidly by the kidneys in the rat.[28] To be effective, it appears that SOD must be present at the time of oxygenation and O_2^- generation and that the effect is rapid.

Superoxide radical-induced cytotoxicity appears to depend largely on the subsequent production of a highly reactive species, the hydroxyl radical (or its redox equivalent) catalyzed by iron, which is probably in the form of an organic iron complex[31] (see Fig. 10). Reactive oxygen species are implicated as the cause of increased permeability of cell membranes of endothelium and epithelium. Increased permeability in cell membranes of tubular epithelial cells or capillary endothelial cells of kidney could lead to disruption of vital transport functions. Membrane damage by reactive oxygen species could occur at the plasmalemma with secondary release of other mediators such as lipid endoperoxides, arachidonic acid, and hydroperoxides. Either these mediators or the initiating species could induce endothelial cell contraction terminating in a macromolecular leak.[32] Hydroxyl free radical has been shown to degrade hyaluronic acid,[33] which is one of the principal constituents of the interstitial matrix and the capillary basement membrane.[35] Oxygen free radical-induced damage to the capillary basement membrane could exacerbate the microvascular leak already established as a result of endothelial cell damage.

Although superoxide radicals might act as primary mediators of ischemic tissue injury, with a more severe or prolonged period of ischemia other factors such as ATP depletion and proteolysis may play a more important role

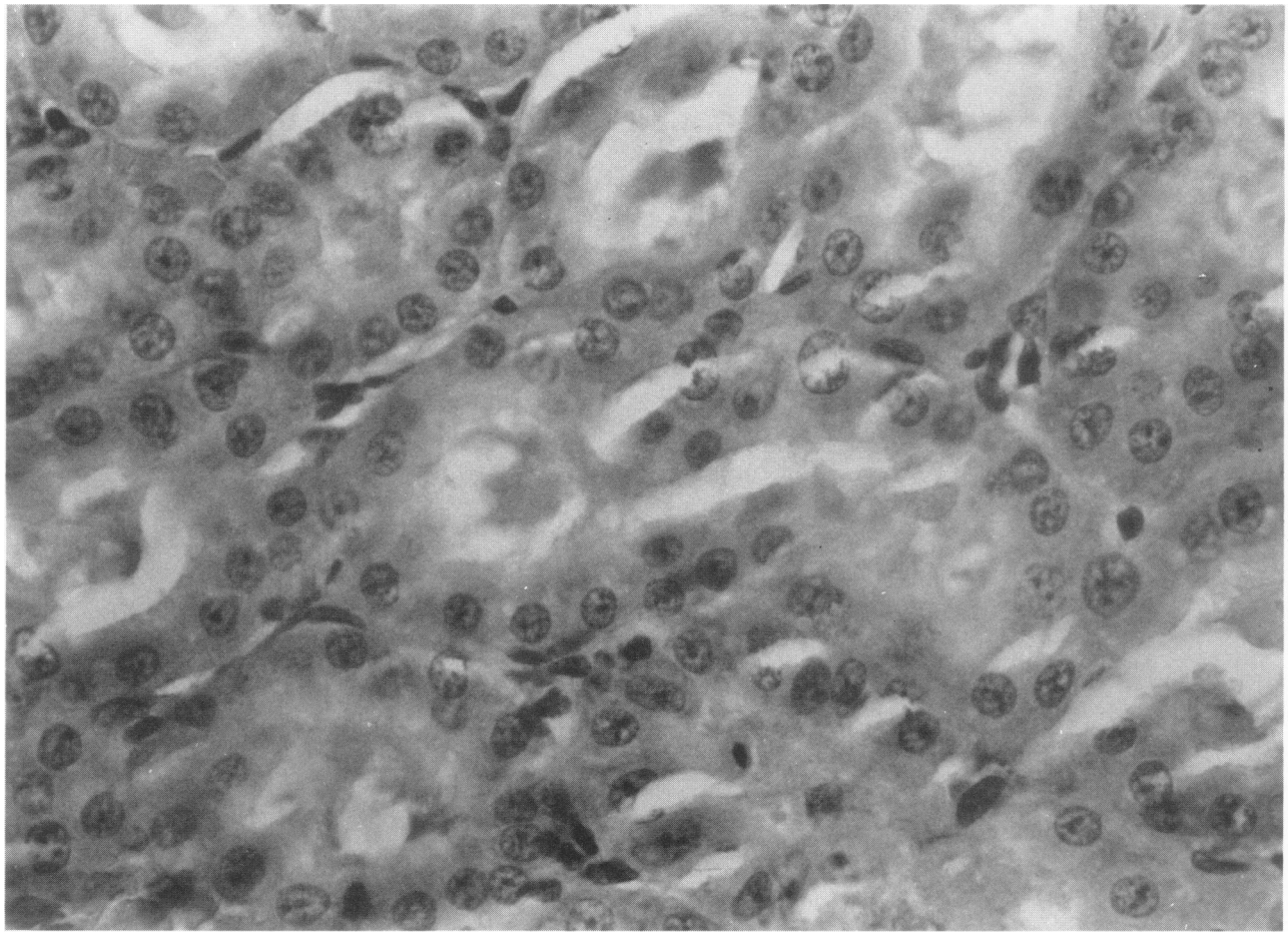

FIG. 7. Section of rat renal cortex taken on postoperative day 3 from the left kidney of an ischemic 45 minute rat treated with superoxide dismutase (6.5 mg/kg) two minutes before reperfusion. Necrosis of isolated cells and mitotic figures are seen. Representative of histopathologic Grade 1. Original magnification ×400.

in the chemical and structural alteration of tissue. These other factors could then negate the protective effects of SOD following severely prolonged renal ischemia similar to that which occurs upon occlusion for 1 hour of the superior mesentery artery.[11]

Allopurinol was employed in several earlier studies to prevent an irreversible loss of purine nucleotides from cells during ischemia.[36–38] During ischemia purine nucleotides are converted to adenosine and guanosine and subsequently degraded to uric acid (Fig. 9). When purines have been catabolized from xanthine to uric acid enzymatically, they are irreversibly lost from the nucleotide pool. The degraded purines are thus no longer available to the cell for the resynthesis of ATP when oxygen delivery is restored. Allopurinol was previously thought to provide protection against ischemic damage by preventing the loss of purine nucleotides from the intracellular nucleotide pool during periods of hypoxic stress *via* inhibition of xanthine oxidase.[38–40] For example, pretreatment with allopurinol significantly improved survival rate and postischemic renal function in animals subjected to 40–120 minutes of ischemia.[40] Allopurinol was also shown to limit infarct size, diminish losses in myocardial contractility, and reduce the incidence of arrhythmias produced by temporary coronary artery ligation.[37,41] Additional support for the purine nucleotide depletion hypothesis was the finding that the concentration of purine nucleotides (ATP, ADP, and AMP) in rat kidneys decreased after 1 hour of ischemia, but when animals were pretreated with allopurinol, the postischemia concentration of these nucleotides was maintained at relatively normal levels.[39,42] While our study cannot completely exclude a role for this proposed mechanism, we suggest that the beneficial effects of allopurinol observed in our model appeared to come from the inhibition of O_2^- production by xanthine oxidase rather than by maintenance of cellular levels of purine nucleotides during ischemia.

Xanthine oxidase is probably the main source of su-

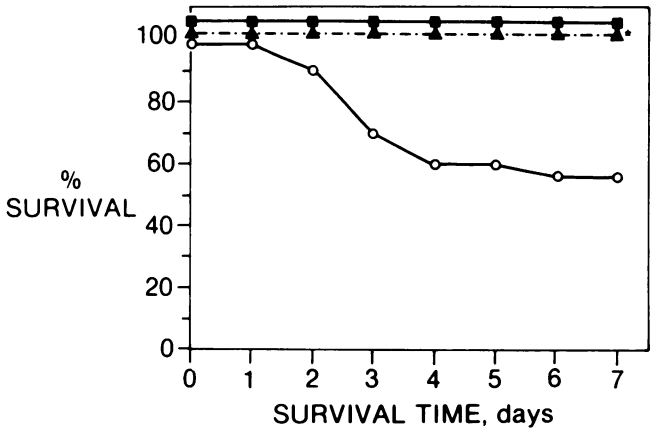

FIG. 8. Per cent survival over a 7-day postoperative period of rats that have undergone 45 minutes of renal ischemia and treatment with allopurinol. Rats in all experimental groups had the right kidney removed. Untreated control (sham-operated) rats with right nephrectomy only (N = 8) (■ — ■); left renal artery occluded for 45 minutes and 5% dextrose in water injected intravenously 2 minutes before reflow (N = 30) (○ — ○); left renal artery occluded for 45 minutes and 100 mg/kg allopurinol injected intravenously 2 minutes before reflow (N = 12) (▲ — ▲).

peroxide radicals when ischemic small intestine is reperfused with well-oxygenated blood.[8,27] The enzyme appears to be present in sufficient quantities in the rat kidney to be potentially an important source of O_2^-.[43] During renal ischemia, tissue levels of hypoxanthine, a substrate for xanthine oxidase, rise rapidly to 10 to 300 times greater than normal.[42–44] Molecular oxygen is supplied in excess during reperfusion. In healthy tissue, the enzyme normally exists as xanthine dehydrogenase (Type D), a form of the enzyme that transfers substrate electrons to NAD^+ rather than to oxygen.[46,48]

(1) Hypoxanthine + NAD^+ + $H_2O \xrightarrow{XD}$

Xanthine + NADH + H^+

XD = Xanthine dehydrogenase (Type D)

(2) Hypoxanthine + $2O_2$ + $H_2O \xrightarrow{XO}$

Xanthine + $2O_2^-$ + $2H^+$

XO = Xanthine oxidase (Type O)

Xanthine dehydrogenase (Type D) is converted to the xanthine oxidase (Type O) form of the enzyme by limited proteolysis during ischemia (Fig. 10). In the nonperfused rat intestine, this proteolytic conversion from dehydrogenase (Type D) to oxidase (Type O) is complete in less than 1 minute.[47–49] The exact mechanism whereby hypoxia initiates this proteolytic conversion is not known. In our study, allopurinol was administered immediately before reflow; therefore, it could not affect purine nucleotide metabolism during the period of ischemia. The protection afforded the rat kidney by allopurinol when administered immediately before reperfusion was similar to that previously observed when allopurinol was administered for prolonged periods to dogs both before and after renal ischemia.[38] These results imply that the conversion of XD to XO in rat kidney under our experimental conditions may be relatively rapid. Because SOD administered immediately before reperfusion provided similar, although somewhat superior protection, against renal dysfunction when compared with allopurinol, both SOD and allopurinol may limit renal injury by similar mechanisms. Allopurinol probably blocks the generation of the superoxide anion by inhibition of xanthine oxidase, and SOD catalytically removes superoxide anions generated by xanthine oxidase by a mechanism similar to that proposed to explain the protective effects of SOD and allopurinol after temporary ischemia in the regional small bowel[26] and the ischemic small intestine.[27]

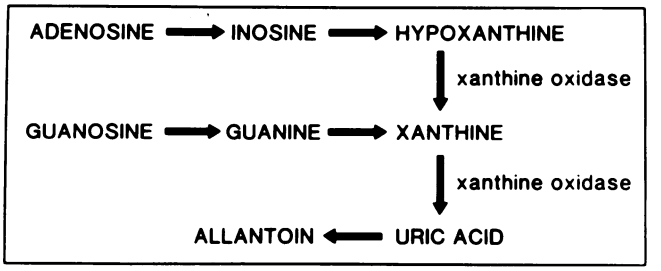

FIG. 9. Scheme depicting the role of xanthine oxidase in the degradation of purine nucleotides to uric acid (modified from reference 50).

If xanthine oxidase were the sole source of the postischemic O_2^- production in the rat kidney, then treatment with allopurinol might be expected to provide protection comparable to, or better than, that provided by SOD. Our

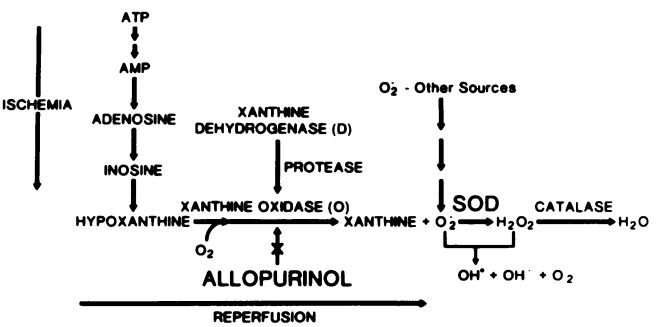

FIG. 10. Scheme depicting the proposed mechanism for the production of superoxide anion free radical (O_2^-) and other reactive oxygen species after transient ischemia followed by reperfusion with oxygenated blood. Allopurinol inhibits the catalytic activity of xanthine oxidase (Type O) and thus the production of O_2^- by this enzyme. Superoxide dismutase catalytically removes O_2^- generated from all possible sources (modified from reference 17).

study, however, shows that SOD is superior to allopurinol in limiting postischemic renal dysfunction. This suggests that O_2^- is generated from other sources in postischemic kidney in addition to xanthine oxidase.

The histopathological changes produced by 45 minutes of ischemia in this study were similar to those previously reported for induced renal injury.[47] Generally, the lesions produced by 45 minutes of renal ischemia were characterized by necrosis of the distal third of the proximal convoluted tubule with a band of necrosis extending across the inner renal cortex. From our study, it is not possible to localize precisely the site of protection by SOD. Reactive oxygen species could damage renal tubular epithelial cells, glomerular mesangial cells, arteriolar endothelial cells, as well as structural components of the basement membrane and interstitium. On the basis of previous studies, the renal cortex appears to be the major site for protection by SOD and allopurinol.[20] Treatment of kidneys with SOD and to a lesser extent with allopurinol appeared to ameliorate the acute morphologic tubular damage produced by 45 minutes of ischemia followed by reperfusion. Although some degree of tubular damage was observed in both groups of animals treated with either SOD or allopurinol, the severity of morphologic tubular damage in these groups was substantially less than in the surviving untreated animals. In fact, kidneys removed from several rats 7 days after 45 minutes of ischemia and treatment with SOD immediately before reperfusion were virtually normal in appearance. Thus, the histologic evidence paralleled and validated the functional data collected from rats treated with SOD and allopurinol.

SOD and allopurinol (unpublished data) both provided protection against renal dysfunction following temporary ischemia of longer duration. This protection was not complete, however. The degree of protection against renal dysfunction was less in animals treated with SOD as the period of ischemia was extended. There are a number of possible explanations for this finding, the most likely being that factors other than oxygen free radicals (*e.g.*, irreversible loss of mitochondrial function and ATP depletion and/or activation of proteolytic enzymes) contribute to renal dysfunction as the period of ischemia is extended. It is not known to what extent SOD can enter cells or what the intracellular distribution of this enzyme might be if some cell entry does occur, although it may not be important in this model that SOD act intracellularly. Despite these factors, SOD and allopurinol provided significant protection following temporary renal ischemia based on improved survival, reduced renal dysfunction, and improved renal tubular morphology. At present, the clinical and therapeutic importance of SOD and allopurinol in acute renal failure and possibly in kidney or other organ transplant is not known; however, these agents show promise by their ability to ameliorate renal dysfunction following temporary renal ischemia and reperfusion.

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