*Biochemical Pharmacology*, Vol. 35, No. 2, pp. 341–345, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00


# EFFECT OF SUPEROXIDE DISMUTASE ON GLOMERULAR NEPHRITIS

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(*Received* 24 *October* 1984; *accepted* 16 *April* 1985)

**Abstract**—The antiinflammatory effect of superoxide dismutase was studied in rats with kidney intoxication induced by the injection of nephrotoxic serum. The urinary excretion of protein was increased significantly by the administration of an intravenous injection of nephrotoxic serum. The daily injection of superoxide dismutase significantly suppressed the increase in urinary excretion of protein. The injection of nephrotoxic serum increased the renal malondialdehyde level by 3-fold compared to the level in the control rats. The daily injection of superoxide dismutase also significantly suppressed the renal malondialdehyde concentration. The change of serum Cu,Zn-superoxide dismutase level and tissue distribution after the intramuscular injection of this enzyme was investigated by immunoassay. Human Cu,Zn-superoxide dismutase localized in kidney exclusively and reached maximum concentration at about 3 hr after the injection. From these results, we propose that superoxide dismutase, which is a scavenger of superoxide anion radicals, inhibits lipid hydroperoxidation in the kidney induced by activated oxygen, and thus protects the renal cells from the damage induced by the injection of nephrotoxic serum.

Activated oxygen radicals which are produced by chemical and biochemical reactions are thought to induce deleterious effects such as lipid peroxidation [1], inflammation [2], carcinogenesis [3], cataracts [4] and atherosclerosis [5], even though the oxygen molecule is essential to life.

Most types of nephritis are known to be caused by immunological mechanisms: (1) binding of heterologous antibodies to glomerular antigens, or (2) adherence of immune complexes produced in the blood vessels [6]. Immunological complex formation stimulates polymorphonuclear leukocytes or macrophages to release superoxide anion radicals. Among activated oxygen species, superoxide anion radical is initially produced and this, in turn, gives rise to other activated oxygen species such as hydroxyl radicals and singlet oxygen. These oxygen radicals have been implicated in biological phenomena. They initiate the lysis of lysosomes and peroxidation of cell membrane lipids.

Therefore, a powerful scavenger of superoxide anion radical would be effective in reducing or preventing nephritis. Superoxide dismutase is a highly potent scavenger of superoxide anion radical and was discovered in bovine erythrocytes by McCord and Fridovich [7].

It has been reported that superoxide dismutase has clear antiinflammatory effects in carrageenan-induced footpad oedema, reversed passive Arthus reaction [8] and immune complex-induced nephritis [9]. Clinically, bovine Cu,Zn-superoxide dismutase has been used under the name of orgotein and yielded good clinical results in rheumatoid arthritis [10]. In this paper, we describe the use of exogeneous superoxide dismutase in glomerular nephritis as an antiinflammatory agent.

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## MATERIALS AND METHODS

*Experimental animals.* Male Wistar rats were obtained from the Shizuoka Agricultural Co. Association of Laboratory Animals (Japan). The animals were provided with a standard laboratory diet and water.

*Preparation of nephrotoxic serum.* Nephrotoxic serum was prepared by the method of Unanue and Dixon [11]. Fresh rat kidney was washed and the medulla removed. Cortex was diced and passed through a 150-mesh stainless steel sieve. The pellet, which was obtained by centrifuging at 300 *g* for 10 min, was resuspended in 10 vol. of physiological saline. This fraction contained glomerular basement membrane (GBM). The suspension was emulsified with an equal volume of Freund's complete adjuvant and administered subcutaneously to female rabbits. The rabbits received three further injections at weekly intervals and were bled a week after the last injection.

Antisera were decomplemented by heat at 56° for 30 min and adsorbed with rat erythrocytes. Antisera obtained as mentioned above (nephrotoxic serum: NTS) were frozen until use.

*Protocol of experiments.* Human Cu,Zn-superoxide dismutase was purified from erythrocytes according to the method of McCord and Fridovich [7]. The specific activity was about 3100 units/mg.

Five rats were injected intravenously with 0.6 ml of NTS (42.4 mg protein/ml) and used as experimental

animals (group I). Five rats received NTS as mentioned above and daily intramuscular injections of 1.8 mg/kg of human Cu,Zn-superoxide dismutase (group II). Another five rats were injected with human Cu,Zn-superoxide dismutase only (group III), while the five control rats received physiological saline only.

*Assay of enzyme activities and other clinical values.* Urine was collected by housing each animal in an individual metabolic cage for 24 hr. Urinary protein concentration was determined by the turbidimetric method [12]. Urinary creatinine concentration was determined by the method of Bonsnes and Taussky [13]. Alkaline phosphatase activity was assayed by the Kind–King method [14]. Inorganic phosphorus concentration was determined by the method of Fiske and Subbarow [15], protein concentration in tissues by the method of Lowry *et al.* [16], and lipid hydroperoxide concentration by the method of Ohkawa *et al.* [17]. Human Cu,Zn-superoxide dismutase concentration in sera after the injection of this enzyme was determined by enzyme immunoassay [18].

*Isolation of polymorphonuclear neutrophils.* Polymorphonuclear neutrophils were prepared by the method of Coupland and Leslie [19]. Wistar rats were used as the source of polymorphonuclear neutrophils. Sixteen hours after the intraperitoneal injection of 25 ml of 1% oyster glycogen solution, peritoneal exudate cells were harvested from rats. The cells obtained were washed and suspended in minimum essential medium.

*Measurement of superoxide anion radical formation.* The determination of superoxide anion radical production was performed by measuring the oxidation of NADH by superoxide anion radicals in the presence of lactate dehydrogenase [20].

## RESULTS

*Time course of changes in urinary excretion.* Rat urinary protein and creatinine were determined. Urinary protein concentrations of control rats averaged 1.24 ± 0.36 mg/mg of creatinine. As shown in Fig. 1, proteinuria occurred in all rats in group I after a lag phase. Twenty-one days after the injection of NTS, urinary protein concentration reached about 10-fold that of control rats and then gradually decreased.

The daily administration of superoxide dismutase significantly suppressed the increase in urinary excretion of protein (Fig. 1, group II). There was no significant difference in urinary protein concentration between rats in group II and control rats. No effect of administration of superoxide dismutase to normal rats was observed (compare group III with the control group).

Rats in group I showed high alkaline phosphatase activity in urine when compared to the control group, as shown in Fig. 2. Rats in groups II and III showed the same level of urinary alkaline phosphatase as the control group. Moreover, there were no significant differences in urinary excretion of inorganic phosphorus among the four groups (Fig. 3).

*Morphological changes in kidney.* Thickening of mesangium, obliteration of capillaries and decrease

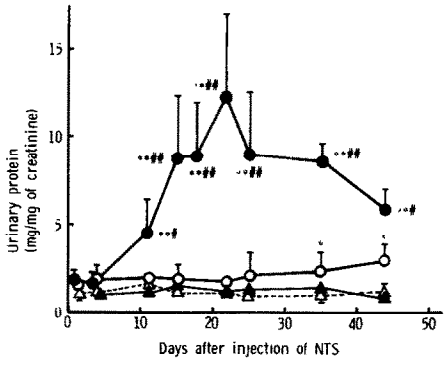


Fig. 1. Time course of change in urinary excretion of protein in rats after injection of nephrotoxic serum. Key: (—●—) group I (NTS only); (—○—) group II (NTS + superoxide dismutase); (—▲—) group III (superoxide dismutase only); (--△--) control; (*) $P < 0.05$ vs control; (**) $P < 0.005$ vs control; (#) $P < 0.01$ vs group II; and (##) $P < 0.005$ vs group II. Vertical bars depict ± S.D.

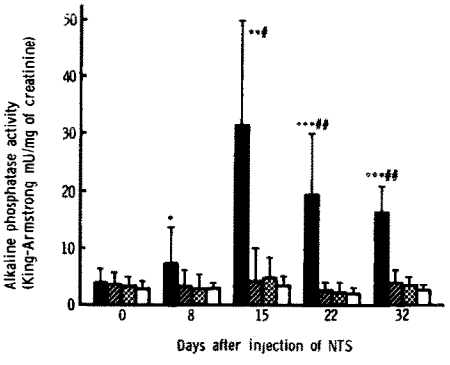


Fig. 2. Change in urinary excretion of alkaline phosphatase after injection of nephrotoxic serum. Key: (■) group I (NTS only); (▨) group II (NTS + superoxide dismutase); (▩) group III (superoxide dismutase only); (□) control; (*) $P < 0.05$ vs control; (**) $P < 0.01$ vs control; (***) $P < 0.005$ vs control; (#) $P < 0.05$ vs group II; and (##) $P < 0.005$ vs group II. Vertical bars depict ± S.D.

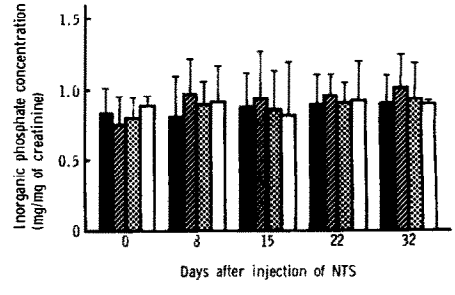


Fig. 3. Change in urinary excretion of inorganic phosphorus after injection of nephrotoxic serum. Key: (■) group I (NTS only); (▨) group II (NTS + superoxide dismutase); (▩) group III (superoxide dismutase only); and (□) control. Vertical bars depict ± S.D.

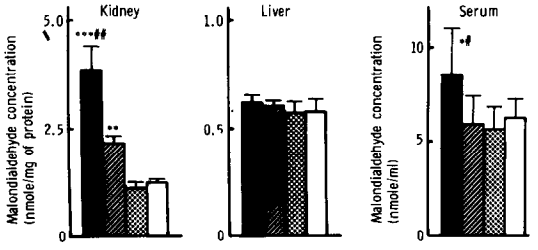


Fig. 4. Lipid hydroperoxide levels in kidney, liver and serum after injection of nephrotoxic serum. Key: (■) group I (NTS only); (▨) group II (NTS + superoxide dismutase); (▩) group III (superoxide dismutase only); (□) control; (*) $P < 0.10$ vs control; (**) $P < 0.05$ vs control; (***) $P < 0.005$ vs control; (#) $P < 0.10$ vs group II; and (##) $P < 0.005$ vs group II. Vertical bars depict ± S.D.

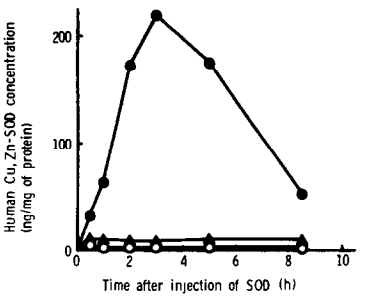


Fig. 6. Human Cu,Zn-superoxide dismutase concentration in kidney (—●—), liver (—○—) and heart (—▲—) of rats following intramuscular administration.

of erythrocytes in capillary lumens were frequently observed. In the kidneys of rats in group II, the morphological changes were slight compared with those in the rats in group I.

*Lipid hydroperoxide levels in tissue and serum.* Because superoxide dismutase catalyzes the dismutation of superoxide anion radical which can trigger free radical reactions leading to the peroxidation of polyunsaturated fatty acid components of membrane lipids, lipid hydroperoxide concentrations in kidney and liver of rats were assayed. As shown in Fig. 4, at 21 days after the injection of NTS, lipid hydroperoxide level in kidneys from group I rats was about 3-fold higher than that from control rats, and the kidneys of rats in group II showed lipid hydroperoxide levels about half those found in kidneys of rats in group I. On the other hand, lipid hydroperoxide levels in liver showed no significant differences among the four groups. Lipid hydroperoxide concentration in sera of rats in group I was significantly higher than that in sera of the other groups.

*Disposition of serum Cu,Zn-superoxide dismutase administered to rats.* The disposition of serum human Cu,Zn-superoxide dismutase after the administration of a single dose of this enzyme was investigated by enzyme immunoassay. Human Cu,Zn-superoxide dismutase concentration in serum reached a maximum level 1 hr after intramuscular injection, and the half-life was 2.87 ± 0.54 hr. On the other hand, Cu,Zn-superoxide dismutase intravenously injected was eliminated immediately from the circulation and its half-life was 10.8 min. Bioavailability of human Cu,Zn-superoxide dismutase administered to glomerulonephritic rats was also investigated. Time to reach maximum level and half-life were almost the same as seen with control rats (Fig. 5).

Tissue distribution of human Cu,Zn-superoxide dismutase after the intramuscular injection was also investigated. Figure 6 shows that Cu,Zn-superoxide dismutase scarcely appeared in liver and heart, but it accumulated in the kidney. The peak of Cu,Zn-superoxide dismutase concentration in the kidney appeared about 3 hr after the intramuscular injection.

*Superoxide anion radical formation by neutrophils.* It is well known that polymorphonuclear neutrophils or macrophages generate superoxide anion radicals when exposed to appropriate stimuli such as immune complexes. The effect of the immune complex formed from GBM (antigen) and NTS (antibody)

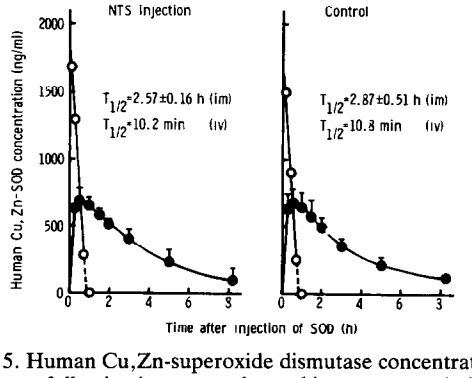


Fig. 5. Human Cu,Zn-superoxide dismutase concentration in serum following intramuscular and intravenous administration. Human Cu,Zn-superoxide dismutase (1.8 mg/kg) was administered to nephritic rats and normal rats. Key: (—●—) intramuscular administration; and (—○—) intravenous administration.

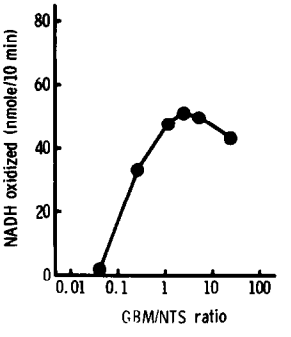


Fig. 7. Superoxide anion radical generation from neutrophils stimulated with glomerular basement membrane (GBM) rich fraction and nephrotoxic serum (NTS) complexes at various ratios.

on the ability of neutrophils to produce superoxide anion radical was investigated. To remove Cu,Zn-superoxide dismutase and Mn-superoxide dismutase present in GBM fractions, GBM fractions were passed through anti-Cu,Zn-superoxide dismutase antibody-conjugated Sepharose 4B and anti-Mn-superoxide dismutase antibody-conjugated Sepharose 4B columns.

As shown in Fig. 7, it was observed that GBM–NTS complexes enhanced the generation of superoxide anion radicals. The amount of superoxide anion radical produced was dependent upon the molar ratio of GBM and NTS, indicating that the formation of antigen–antibody complex at an optimum ratio may be required for the stimulation of superoxide anion radical formation.

## DISCUSSION

The antiinflammatory effect of superoxide dismutase on glomerular nephritis has been investigated. Rats injected with single doses of NTS developed nephritis with high proteinuria. Daily administration of superoxide dismutase by intramuscular injection significantly suppressed the increase of urinary protein excretion.

Nephrotoxic serum nephritis proceeds in two phases. The first phase occurs immediately after the injection of NTS. During this period, heterologous nephrotoxic serum reacts with glomerular antigens and, subsequently, accumulation of leukocytes in glomeruli occurs. It has been reported that leukocytes accumulate in large numbers in the glomeruli in the first phase and that the amount of proteinuria is proportional to the number of leukocytes [21]. The secondary phase starts several days after the injection and is due to host response, in other words, by the deposit of rat antibodies against rabbit nephrotoxic serum in the glomeruli. As shown in Fig. 1, in this stage, proteinuria increased rapidly. This secondary period terminated about 3–4 weeks after the injection of NTS, presumably at the time that rats stopped producing antibodies against the rabbit nephrotoxic serum.

Neutrophils and macrophages are known to generate superoxide anion radicals when exposed to appropriate stimuli [22]. The binding of immune complexes to Fc receptors on the cell surface generates stimuli which induce superoxide anion radical generation by NADPH-oxidase. These superoxide anion radicals and other oxygen radicals are known to induce lipid peroxidation.

As shown in Fig. 4, the renal lipid hydroperoxide level was stimulated significantly by the injection of NTS, while the content of lipid hydroperoxide in liver remained constant. Administration of superoxide dismutase also resulted in suppression of the renal lipid hydroperoxide level. Goldstein and Weissman [23] and Dobretsov *et al.* [24] reported that rigidity and permeability of membranes are increased by lipid peroxidation. From the results mentioned above, the inhibition of lipid peroxidation of GBM by superoxide dismutase suppresses the elimination of protein and the exudation of leukocytes. Moreover, superoxide dismutase may suppress the release of lysosomal proteinase by the inhibition of lipid peroxidation of plasma membranes or lysosomal membranes of leukocytes and macrophages. On the other hand, Ōyanagui reported that superoxide dismutase inhibited the release of chemoattractants [25]. These results strongly suggest that superoxide dismutase administration effectively dismutates the superoxide anion radical produced by immunoreaction in kidney and that superoxide dismutase is a potent agent in preventing the damage from active oxygen.

In mammalia, as superoxide dismutase concentration in serum is very low [16], superoxide anion radicals, which are produced by NADPH oxidase located on the plasma membrane of polymorphonuclear neutrophils, cannot be eliminated by superoxide dismutase. When superoxide dismutase was injected intramuscularly or intravenously, the level of this enzyme in serum increased, as shown in Fig. 5. While exogenous superoxide dismutase was cleared rapidly from circulation after intravenous injection, it remained longer in circulation after intramuscular injection. In addition to these results, the fact that the exogenous superoxide dismutase tends to accumulate in the kidney is convenient for the antiinflammatory effect of superoxide dismutase. Probably superoxide dismutase inhibits a chain reaction leading to inflammation and slows down the self-perpetuating process of tissue damage. Fortunately, superoxide dismutase has proven to be an extremely weak immunogen, so that the antiinflammatory effect is long-lasting and causes no tissue toxicity.

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