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Does superoxide underlie the pathogenesis of hypertension?

(oxygen toxicity/superoxide dismutase fusion protein/heparin/endothelium-derived relaxing factor)

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ABSTRACT **Although active oxygen species play important roles in the pathogenesis of various diseases, the molecular mechanism for oxygen toxicity in vascular diseases remains to be elucidated. Since endothelium-derived relaxing factor (EDRF) is inactivated by superoxide radicals *in vitro*, oxidative stress in and around vascular endothelial cells may affect the circulatory status of animals. To study the role of superoxide radicals and related enzymes, such as superoxide dismutase (SOD), in vascular diseases, we have developed a fusion protein (HB-SOD) consisting of human Cu/Zn-type SOD and a C-terminal basic peptide with high affinity for heparan sulfate on endothelial cells. When injected intravenously, HB-SOD bound to vascular endothelial cells, underwent transcellular transport, and localized within vascular walls by a heparin-inhibitable mechanism. The blood pressure of spontaneously hypertensive rats (SHR) but not normal animals was decreased significantly by HB-SOD. Heparin inhibited the depressor effect of HB-SOD. In contrast, native SOD had no effect on blood pressure of either SHR or normal rats. Neither H_2O_2-inactivated HB-SOD nor the C-terminal heparin-binding peptide showed such a depressor effect, suggesting that the catalytic function of HB-SOD is responsible for its depressor action. To know the source of superoxide radicals, we determined xanthine oxidase activity in the aorta and uric acid levels in the plasma. Although no appreciable difference in xanthine oxidase activity was found between the two animal groups, uric acid levels were significantly higher in SHR than in normal rats. Oxypurinol, a potent inhibitor of xanthine oxidase, also decreased the blood pressure of SHR but not of normal rats. These findings indicate that superoxide radicals in and around vascular endothelial cells play critical roles in the pathogenesis of hypertension of SHR.**

Reactive oxygen species play critical roles in the pathogenesis of various diseases, such as cardiovascular injury associated with circulatory disturbance (1, 2). Many factors, such as endothelin and endothelium-derived relaxing factor (EDRF), affect the circulatory status of animals by modulating vascular resistance. Nitric oxide (NO) and NO-generated compounds account for the biological actions of EDRF (3, 4). EDRF is synthesized predominantly in vascular endothelial cells and is transferred to smooth muscle cells, where it facilitates cGMP generation leading to vasodilatation. Superoxide radicals can inactivate NO and, hence, EDRF-dependent relaxation of aortic rings and endothelium-denuded aortas was enhanced by Cu/Zn-type superoxide dismutase (SOD) (5, 6). Based on such *in vitro* experiments, superoxide radicals have been postulated to affect vascular resistance by inactivating EDRF. Since intravenously injected Cu/Zn-SOD lacks tissue-specific localization and disappears rapidly from the circulation, predominantly due to glomerular filtration (7), the use of natural SOD for *in vivo* study is highly limited. Hence, the possible involvement of superoxide radicals in the regulation of vascular resistance *in vivo* remains to be studied. To determine whether superoxide functions as a vasoactive metabolite *in vivo*, SOD should be targeted to the site(s) of action of superoxide radicals and EDRF in resistant arteries. We have constructed a fusion gene that contains the cDNA for human Cu/Zn-SOD and encodes a C-terminal basic domain with high affinity for heparin-like proteoglycans (8, 9). The fusion gene product, HB-SOD, expressed in yeast has high affinity for heparan sulfate on vascular endothelial cells. Okamoto and Aoki (10) described a mutant strain of spontaneously hypertensive rats (SHR). SHR have been used as an animal model for essential hypertension. The present work describes immunocytochemical localization of HB-SOD in the arteries and the effect of HB-SOD on blood pressure of SHR and normal rats.

MATERIALS AND METHODS

Materials. Heparin and oxypurinol were purchased from Nakarai Tesque (Kyoto) and Sigma, respectively. Human Cu/Zn-SOD and the heparin-binding peptide (PGIWERQAREHSERKKRRRESECKAA) were obtained from Suntory (Osaka). HB-SOD was purified from yeast lysate (9); the specific activity of HB-SOD was 2750 units/mg of protein, as determined by the cytochrome *c* method (11). Inactivation of HB-SOD was carried out by incubating the enzyme with 0.1 M H_2O_2 at 37°C for 3 hr. After extensive dialysis against saline solution (0.9% NaCl), the enzyme samples were used for experiments. Protein concentration was determined by the method of Lowry *et al.* (12) with human SOD as the standard. Antiserum against human SOD was produced in New Zealand White rabbits as described (13). All reagents used were of analytical grade.

Animals and Blood Pressure Measurements. Male SHR and Wistar Kyoto rats (200 g) were fed laboratory chow and water *ad libitum* and put in a thermostatted chamber (37°C) 30 min before experiments. Under light ether anesthesia, animals were intravenously injected with various compounds dissolved in 0.2 ml of saline solution. Then, blood pressure of conscious animals was monitored from the tail artery by a sphygmomanometer (PS-200A; Riken Kaihatsu, Tokyo).

Enzymes. After exsanguination of animals, the aorta was perfused with ice-cold saline. After homogenization of the excised aorta in 5 volumes of ice-cold phosphate-buffered saline (pH 7.4) in a Polytron homogenizer (Brinkmann), the homogenate was centrifuged at 13,000 × *g* for 10 min. SOD and xanthine oxidase activities in the supernatant were determined as described (11, 14).

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Abbreviations: EDRF, endothelium-derived relaxing factor; SHR, spontaneously hypertensive rat(s); SOD, superoxide dismutase; HB-SOD, SOD fusion protein with a heparin-binding C-terminal basic domain.

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Immunohistochemical Studies. At the indicated times after administration of SOD or HB-SOD (25 mg/kg of body weight), rats were exsanguinated and the abdominal aorta was excised. The cryosections of the artery were prepared and immunostained using specific anti-SOD antiserum and horseradish peroxidase-conjugated anti-rabbit IgG antibody (15).

RESULTS

Previous studies in this laboratory revealed that HB-SOD bound to endothelial cells of the artery by a heparin-inhibitable mechanism (8, 9). To know the *in vivo* fate of endothelial cell-associated HB-SOD, immunohistochemical studies were performed with the abdominal aorta (Fig. 1). One minute after administration of HB-SOD to normal rats, examination of the aorta revealed that the enzyme was localized predominantly in the endothelium. After 10 min, HB-SOD was localized both in endothelial cells and in tunica interna and elastica interna. HB-SOD also localized in and around endothelial cells of arterioles, major sites for determining vascular resistance (data not shown). Under identical conditions, natural SOD did not localize in vascular walls. When HB-SOD was administered with heparin (500 units per rat), neither binding to endothelial cells nor arterial localization of the enzyme was seen. Under identical conditions, HB-SOD also localized similarly in the aorta of SHR by a heparin-inhibitable mechanism (data not shown).

To determine whether superoxide radicals in and around arterial endothelial cells affect vascular resistance, the effect of HB-SOD on blood pressure was observed in conscious SHR and normal rats. The blood pressure of SHR but not normal rats decreased by about 50 mmHg (1 mmHg = 133 Pa) within 5 min after administration of HB-SOD, without a decrease in the heart rate (Fig. 2). The depressor action of HB-SOD continued for up to 40 min. In contrast, SOD failed to reduce blood pressure in both animal groups. When administered with heparin, HB-SOD failed to decrease the blood pressure of SHR (Fig. 3). Since basic proteins and peptides, such as protamine, often decrease blood pressure nonspecifically (16), the C-terminal basic domain of HB-SOD may possibly be responsible for the depressor action of the enzyme. However, the depressor effect of HB-SOD was seen only in SHR. In contrast, protamine (10 mg/kg) decreased the blood pressure of both SHR and normal rats by 15–20% for 30 min (data not shown). Furthermore, neither H_2O_2-inactivated HB-SOD nor the C-terminal heparin-binding peptide affected the blood pressure of SHR. These results indicate that catalytic function of HB-SOD might be responsible for the decrease in blood pressure of SHR.

Since HB-SOD decreased blood pressure of SHR but not normal rats, the rate of superoxide production in the resistance artery may be higher in the former than in the latter. Alternatively, the arterial activity to degrade superoxide radicals may be lower in SHR than in normal animals. Superoxide production in vascular endothelial cells may involve xanthine oxidase, which is highly enriched in these cells (17). To test the possible involvement of xanthine oxidase in the pathogenesis of hypertension, blood pressure was monitored before and after administration of oxypurinol, a potent inhibitor of xanthine oxidase (18). As shown in Fig. 4, oxypurinol also decreased blood pressure of SHR for about 60 min without decreasing the heart rate. In contrast, oxypurinol failed to decrease blood pressure of normal rats. Thus, levels of xanthine oxidase and/or its substrate may possibly be higher in SHR than in normal animals.

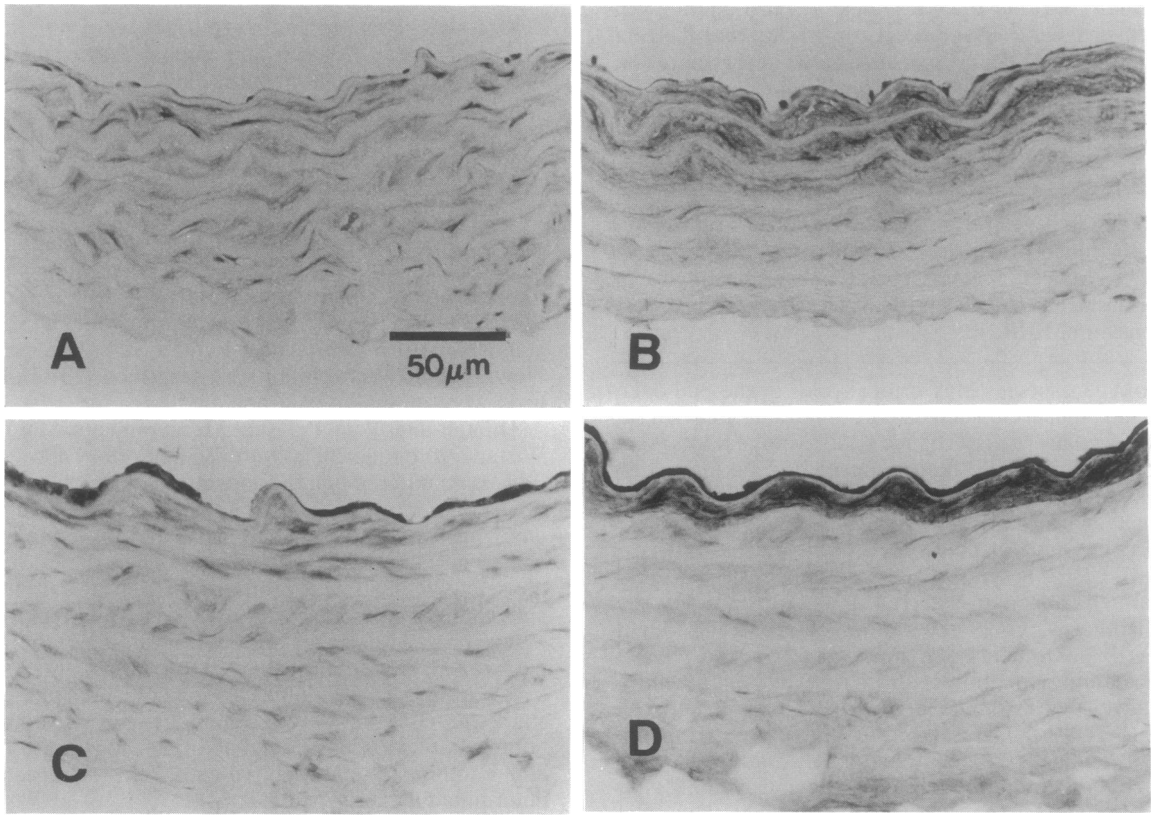

FIG. 1. Immunohistochemical localization of HB-SOD. One and ten minutes after administration of either SOD or HB-SOD (25 mg/kg of body weight) to normal rats (200 g), the abdominal aorta was excised. The cryosections of the aorta were prepared and immunostained (15) using specific antiserum against human SOD and horseradish peroxidase-conjugated anti-rabbit IgG antibody. (*A*) Control group. (*B*) Ten minutes after SOD injection. (*C*) One minute after HB-SOD injection. (*D*) Ten minutes after HB-SOD injection. Data show a typical experiment using three animals.

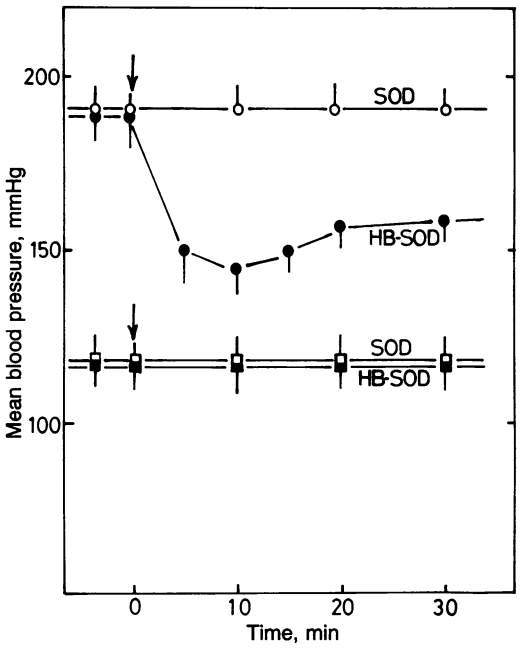

FIG. 2. Effect of HB-SOD on blood pressure of SHR and normal rats. Conscious animals were put in thermostatted cages (37°C) and the blood pressure was determined in the tail artery using a programmable sphygmomanometer (PS-200A; Riken Kaihatsu, Tokyo). Under light ether anesthesia, either SOD (open symbols) or HB-SOD (closed symbols) was administered (25 mg/kg) intravenously to SHR (circles) and normal rats (squares). Data show mean ± SD derived from 10–16 experiments. Arrow shows the time of SOD or HB-SOD injection.

To study the mechanism by which HB-SOD and oxypurinol decreased the blood pressure in SHR but not in normal rats, arterial activity of xanthine oxidase and plasma levels of uric acid, a major metabolite of xanthine, were determined. The plasma level of uric acid was significantly higher in SHR than in normal rats (Table 1). However, no significant difference in arterial xanthine oxidase activity was found between the two animal groups. SOD activity in the aorta of SHR was also similar to that of normal rats.

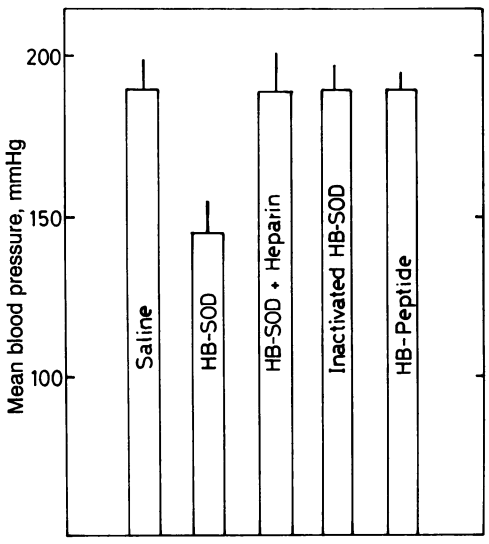

FIG. 3. Effect of HB-SOD and related compounds on blood pressure of SHR. Under the same conditions as in Fig. 2, blood pressure of SHR was determined 10 min after administration of either 0.2 ml of saline, HB-SOD (25 mg/kg), HB-SOD and heparin (2000 units/kg), H_2O_2-inactivated HB-SOD, or an equimolar amount of heparin-binding (HB) peptide. Data show mean ± SD derived from 6–16 experiments.

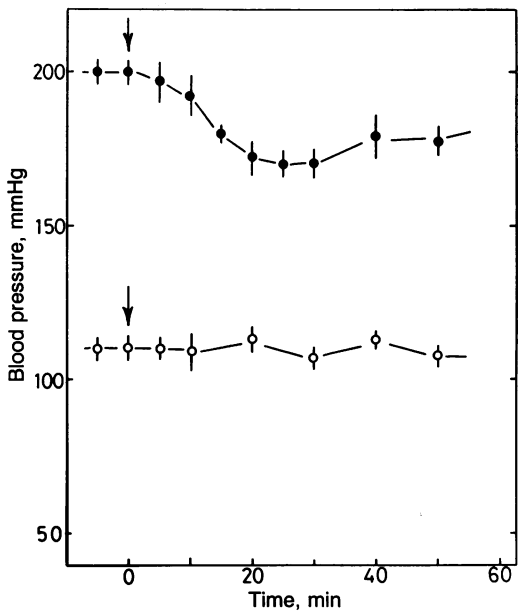

FIG. 4. Effect of oxypurinol on blood pressure of SHR and normal rats. Blood pressure of SHR (●) and normal rats (○) was measured before and after intravenous administration of oxypurinol (17 mg/kg). Other conditions were the same as in Fig. 2. Data show mean ± SD derived from six animals.

DISCUSSION

The present work demonstrates that circulating HB-SOD binds to vascular endothelial cells, undergoes transendothelial transport, and localizes within arterial walls. The mechanism for transendothelial transport of HB-SOD is not known. In this context, Karnovsky (20) reported the presence of a vesicular mechanism for transport of circulating macromolecules across endothelial cells. Hence, vascular endothelial cells may take up circulating proteins nonspecifically by fluid-phase endocytosis and transfer them into contraluminal space. However, such a fluid-phase endocytosis might not account for the transendothelial movement of HB-SOD, since heparin inhibited both binding and transport of the enzyme. That natural SOD, which lacks affinity for the endothelial cell surface, failed to undergo transendothelial movement is consistent with this notion. Thus, binding to heparin-like proteoglycans on the cell surface might be a prerequisite for transendothelial movement of HB-SOD. The mechanism for adsorptive endocytosis and transcellular movement of HB-SOD across vascular endothelial cells should be studied further.

HB-SOD significantly decreased the blood pressure of SHR by a heparin-inhibitable mechanism. Since neither H_2O_2-inactivated HB-SOD nor the C-terminal heparin-

Table 1. Levels of enzymes in aorta and of uric acid in plasma

Rats	Uric acid	Xanthine oxidase	SOD
Normal	3.5 ± 1.0	0.024 ± 0.004	1.50 ± 0.49
SHR	6.2 ± 2.1	0.022 ± 0.005	1.88 ± 0.55

Under light ether anesthesia, blood samples were obtained from the left femoral vein into heparinized tubes. Then, animals were exsanguinated and the aorta was excised. Enzyme activities in the aorta (units/g of protein) were determined as described in the text. Uric acid levels in plasma (μg/ml) were determined as described previously (19). Data show mean ± SD derived from six animals.

binding peptide decreased the blood pressure of SHR, the catalytic activity of HB-SOD might be required for its depressor action. Thus, hypertension of SHR may be classified as so-called "free radical disease" in which superoxide radicals in and around vascular endothelial cells might play important roles.

Theoretical explanations for superoxide-dependent hypertension of SHR may include increased superoxide production, decreased SOD activity, and decreased EDRF synthesis in resistance arteries. In this context, Aisaka *et al.* (21) reported that N^G-monomethyl-L-arginine, an inhibitor of EDRF/NO synthase (22, 23), increased the blood pressure of both normal rats and SHR; the pressor action of the inhibitor was more marked in SHR than in normal rats. This observation suggests that the vascular mechanism for EDRF synthesis also functions efficiently in SHR. Superoxide production in vascular endothelial cells may involve xanthine dehydrogenase/oxidase, which is highly enriched in these cells (17). However, no significant differences in aortic activities of SOD and xanthine oxidase were found between the two animal groups (Table 1). It should be noted that SOD occurs ubiquitously in all types of cells, whereas xanthine oxidase in blood vessels is localized predominantly in endothelial cells. Since the population of endothelial cells within the aorta is less than that of other cells, such as smooth muscle cells, relative activity of xanthine oxidase to SOD within vascular endothelial cells would be higher than that in Table 1. In fact, cultured endothelial cells are reported to have 12.6 milliunits and 4 units of xanthine dehydrogenase/oxidase and SOD per mg of protein, respectively (24, 25). Immunohistochemical studies revealed that the amount of xanthine oxidase in endothelial cells is higher in capillaries and small vessels than in large vessels (26). Furthermore, endothelial cells produce superoxide radicals (27) and oxidize lipoproteins extracellularly by a SOD-inhibitable mechanism (28). Thus, superoxide radicals may come out of vascular endothelial cells and react with EDRF in and around these cells. Since the vascular mechanism for contraction and relaxation is principally the same with large and small arteries, the aorta has been used for *in vitro* study of the mechanism of vascular relaxation. However, arterioles rather than the aorta are the predominant site for determining blood pressure. Since the ratio of xanthine oxidase to SOD in endothelial cells of arterioles might be different from that of the aorta, the activity of the two enzymes should be determined directly with arterioles. Since many different units have been used for SOD activity and none of these is an international unit, one should be careful in comparing the ratio of xanthine oxidase to SOD in various tissues.

The finding that the blood pressure of SHR but not normal animals was decreased markedly by oxypurinol is consistent with the hypothesis that xanthine oxidase-generated superoxide radicals may play critical roles in the pathogenesis of hypertension of SHR. Although xanthine oxidase activity in the aorta of SHR was similar to that of normal rats, plasma levels of uric acid were significantly higher in the former than in the latter. Thus, the rate of degradation of purine nucleotides to xanthine might be higher in SHR than in normal rats. Alternatively, the rate of degradation and/or urinary excretion of uric acid may be lower in SHR than in normal rats. The mechanism for the increase in plasma levels of uric acid in SHR should be studied further.

It is not known whether superoxide radicals also underlie the pathogenesis of essential hypertension in humans. However, in patients with essential hypertension, hyperuricemia is often seen, and allopurinol, a xanthine oxidase inhibitor, decreases blood pressure in these patients (29, 30). Furthermore, hypotension is one of the frequently observed side effects of allopurinol (31). Thus, superoxide radicals and related metabolites may underlie the pathogenesis of certain type(s) of hypertension in human subjects.

Apart from the possible involvement of reactive oxygen species in the etiology of hypertension, HB-SOD, which has high affinity for cell surface heparan sulfate and undergoes transendothelial movement, may have therapeutic potential for vasogenic diseases in which superoxide radicals in and around vascular walls play critical roles.

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