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INHIBITION OF CARRAGEENAN-INDUCED PAW EDEMA BY SUPEROXIDE DISMUTASE THAT BINDS TO HEPARAN SULFATES ON VASCULAR ENDOTHELIAL CELLS

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Abstract—The effect of heparin-binding superoxide dismutase (HB-SOD), a fusion gene product consisting of human Cu/Zn-SOD and a C-terminal basic domain with high affinity for heparin-like proteoglycans, was examined on carrageenan-induced paw edema in mice and rats. When injected intravenously to mice just before carrageenan, HB-SOD suppressed significantly paw edema. ED_{30} of HB-SOD (1000 units/kg) was markedly lower than that of SOD (bovine free Cu/Zn-SOD, 7000 units/kg). When HB-SOD was administered with heparin (500–2000 units/kg), edema was suppressed more markedly than by HB-SOD alone. In contrast, the suppressive action of SOD was decreased by heparin. HB-SOD also suppressed carrageenan paw edema in rats with an ED_{30} of 2500 units/kg which was also obtained by SOD. Heparin prolonged significantly the duration of HB-SOD suppression of edema. The inhibitory effect of HB-SOD alone disappeared within 5 hr of injection, while more than 80% of the effect remained at this time when HB-SOD has been injected with 1000 units/kg of heparin. Heparin failed to enhance the anti-inflammatory effect of SOD under any of the conditions tested and heparin alone showed no suppression up to 5 hr after injection. HB-SOD might permit studies on pathophysiological events in and around vascular endothelial cells where reactive oxygen species play critical roles.

The anti-inflammatory effect of Cu/Zn-type superoxide dismutase (SOD) was observed for the first time in 1976 in rat carrageenan paw edema [1]. However, repeated injection was required to obtain effective suppression due predominantly to rapid excretion of SOD into urine. To overcome such a frustrating situation, several types of SOD derivative have been synthesized such as polyethylene glycol-conjugated SOD [2], liposomal SOD [3] and a hybrid SOD (SM-SOD) that circulates bound to albumin and accumulates in tissues where pH is decreased [4]. Although, small amounts of SOD can be taken up by cells [5], it does not seem to be essential for the anti-inflammatory action because cells have high levels of endogenous SOD. Marklund [6] found extracellular SOD (EC-SOD) of large molecular weight and suggested that the enzyme was important for protecting blood vessels from oxygen toxicity. EC-SOD has three isozymes, A, B and C that show no, weak and high affinity for heparin, respectively [7]. Isozyme A has a similar structure to that of SOD, while EC-SOD B and C both have a positively charged C-terminal domain that involves three lysyl and six arginyl residues. Recombinant EC-SOD C binds to vascular endothelial cells by a heparin-inhibitable mechanism [8]. Unfortunately, a large scale expression of EC-SOD C is difficult, presumably due to the nature of its glycoprotein. Hence, a report on the anti-inflammatory effect of EC-SODs *in vivo* is not yet available. Heparin suppressed ischemic paw edema and carrageenan-induced paw edema in mice [9, 10]. Although the specific activity of administered SOD from different species was the same, a difference in activity was revealed in the suppression of tissue injury in various inflammatory models [11, 12]. A similar tendency was also reported by Michelson and Puget [13] but the reason why SOD from different species shows a corresponding difference in anti-inflammatory action remains to be elucidated. Since the half-life of hazardous oxygen species is extremely short and these species react rapidly with biomolecules, a relatively high concentration of SOD on or near the endothelial cell surface might favor the enzyme to decrease oxygen toxicity in vasogenic tissue injury. Preparation of a hybrid SOD (HB-SOD) with high affinity for heparin was aimed at targeting human-type SOD to heparin-like proteoglycans on the vascular endothelial cell surface [14]. The primary structure of recombinant HB-SOD is identical to human SOD except that the C-terminal three amino acids are replaced with the basic domain of the C-terminal 29 amino acids of EC-SOD C. In our preliminary experiments [14], HB-SOD suppressed carrageenan-induced paw edema and cold-induced brain edema in rats. The present work describes in detail the effect of HB-SOD on carrageenan-induced paw edema in mice and rats and also the therapeutic significance of heparin coinjection.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (270–300 g)

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and male ddY mice (28–35 g) were obtained from SLC Co., Shizuoka. Animals were kept in an air-conditioned room (22–25°, 50–60% humidity, 7a.m.–7p.m. light/7p.m.–7a.m. dark) for at least 2 days before experiments. They were tested in the same condition without fasting.

Assay. Carrageenan solution (1%) in saline was injected into right foot pads of mice (35 μL). Microinjector for HPLC sample supplier was used for carrageenan. In the primary test, the paw thickness was measured for up to 24 hr after injection with different doses of carrageenan. The paw thickness at 4 hr after carrageenan (1%) injection was found to be the best for determining the SOD effect. Carrageenan-injected right hind paw of control was 2.80–3.20 mm and non-injected left hind paw was 1.80–2.05 mm. Suppressions of paw swelling by SOD (0.5 mL saline/30 g body weight, i.v.) was calculated from these values. Five or six mice were used for the control group and four for the SOD-treated group; the experiment was repeated three times (12 mice in total for one dose).

Carrageenan (1%) was injected into right foot pads of rats (100 μL) with a 0.5 mL injector. Carrageenan-injected right hind paw was 2.85–3.20 g in control while non-injected left hind paw was 1.90–2.10 g. Paw swelling in control was calculated as the average of five or six rats. Paw weight gain of each of the SOD-treated (1.0 mL saline/300 g body weight, i.v.) rats was obtained and its suppression was calculated as in the case of mice. Three rats were used for one dose and each experiment was repeated three times (nine rats in total).

Chemicals. Carrageenan (type IV, λ-type) and bovine Cu/Zn-SOD (3000 units/mg solid) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Heparin sodium (178.9 units/mg, porcine intestinal mucosa) was the product of the Nakarai Chemical Co. (Kyoto, Japan). Heparin-binding superoxide dismutase (HB-SOD) was produced as described by Inoue *et al.* [14]. The molecular weight of dimeric HB-SOD was 37.9 kDa (1.21-fold of cytosolic Cu/Zn-SOD). Enzymatic activity was 4500 units/mg protein as determined by the standard cytochrome *c* method of McCord and Fridovich.

RESULTS

Carrageenan-induced paw edema in mice

Figure 1 shows that both SOD and HB-SOD inhibited carrageenan-induced paw edema in mice. HB-SOD suppressed the edema seven times more potently than did SOD, when ED_{30} values were compared. This was consistent with our previous observation [10] that a fairly large dose of SOD is required for the suppression of carrageenan paw edema in rats. As injection of heparin alone also suppressed dose-dependently paw edema, especially in mice [9], when HB-SOD was administered with heparin, suppression occurred in an additive manner (Fig. 2). In contrast, heparin reversed the suppressive effect of SOD.

Carrageenan-induced paw edema in rats

To test whether the effects of HB-SOD and heparin are observed in species other than mice, the

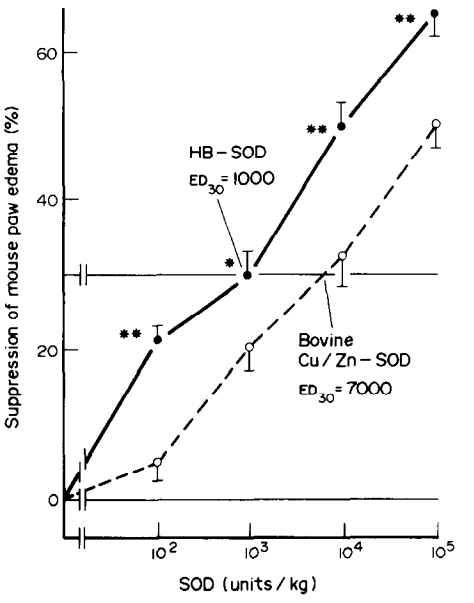

Fig. 1. Dose–response curve of suppression of mouse carrageenan paw edema by SOD. SOD was injected (i.v.) just before carrageenan. Vertical line represents standard error of mean (SEM) of 12 mice (three separate experiments using four mice). Suppression (%) by HB-SOD was analysed statistically against that by bovine free Cu/Zn-SOD (** $P < 0.01$; * $P < 0.05$).

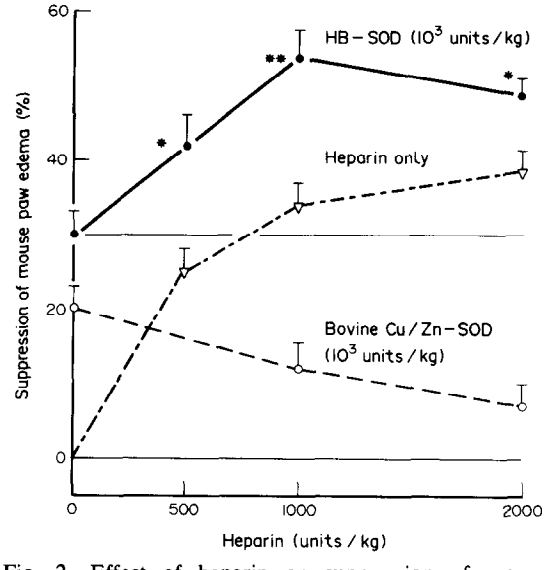

Fig. 2. Effect of heparin on suppression of mouse carrageenan paw edema by SOD. SOD and heparin mixture was injected (i.v.) just before carrageenan. One point represents mean of 12 mice, as for Fig. 1. Suppression (%) by HB-SOD + heparin minus heparin alone was analysed statistically against that by a corresponding dose of Cu/Zn-SOD + heparin minus heparin alone. (** $P < 0.01$; * $P < 0.05$). Suppression (%) by HB-SOD and by Cu/Zn-SOD was also significantly different from heparin alone ($P < 0.01$).

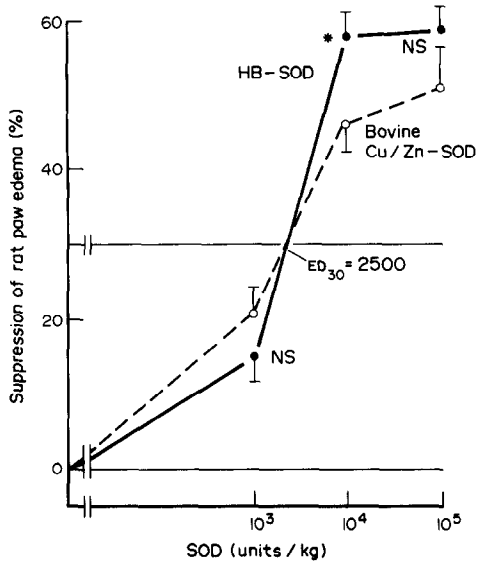

Fig. 3. Dose–response curve of suppression of rat carrageenan paw edema by SOD. SOD was injected (i.v.) just before carrageenan. Vertical line represents standard error of mean (SEM) of nine rats (three separate experiments using three rats). Suppression (%) by HB-SOD was analysed statistically against that by Cu/Zn-SOD (** $P < 0.01$; * $P < 0.05$; NS, not significant).

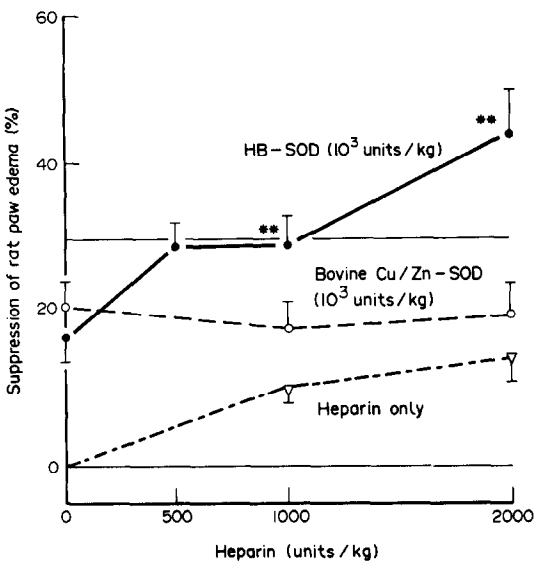

Fig. 4. Effect of heparin co-injection with HB-SOD or bovine free Cu/Zn-SOD on rat carrageenan paw edema. SOD and heparin mixture was injected (i.v.) just before carrageenan. One point represents mean suppression (%) by HB-SOD + heparin minus heparin alone was statistically analysed against that by corresponding dose of Cu/Zn-SOD minus heparin alone (** $P < 0.01$). Suppression (%) by HB-SOD + 2000 units/kg heparin was also significantly different ($P < 0.01$) from that by 2000 units/kg heparin alone.

suppressive effect of SOD and HB-SOD on paw edema was determined in rats. Figure 3 shows that both SOD and HB-SOD suppressed carrageenan-induced paw edema dose-dependently without a difference between their potencies. Though heparin alone suppressed paw edema, this suppression was not remarkable in rats compared with that in mice (Fig. 4). When HB-SOD was administered with heparin, the suppression occurred more markedly than when each was injected alone. The inhibitory action of HB-SOD and heparin appeared to be additive. However, the suppressive effect of SOD remained unchanged even if the enzyme was injected with a fairly large dose of heparin.

To elucidate the mechanism for the different effects of heparin on the suppressive action of HB-SOD and SOD, time-dependent changes in the effect of heparin were observed with two enzymes (Fig. 5). Kinetic analysis revealed that the suppressive effect of both SOD and HB-SOD had disappeared almost completely 5 hr after injection. However, when injected with 1000 units/kg of heparin, the activity of HB-SOD in suppressing paw edema decreased only by about 20%, even 5 hr after administration. The same dose of heparin had no appreciable effect on carrageenan-induced paw edema. Time-dependent changes in the suppressive effect of SOD were not influenced by heparin at any of the time points tested.

DISCUSSION

Since naturally occurring SOD has a short half-life in the circulation (4–6 min), various types of chemically modified SOD have been synthesized to increase the half-life and/or the affinity of the enzyme for cell surface membranes [2–4]. Using such SOD derivatives with prolonged *in vivo* half-lives, inhibition of oxidative tissue injury in various disease models has been demonstrated [4, 14]. The present work shows also that HB-SOD with affinity for heparin-like proteoglycans on the vascular endothelial cell surface has a more potent inhibitory effect on carrageenan-induced paw edema in mice than does SOD. Thus, superoxide radical and/or its reactive metabolite(s) at or near vascular endothelial cells might play critical roles in carrageenan-induced paw edema.

As heparin has multiple functions, such as inhibition of coagulation, release of heparin-binding plasma proteins and enzymes, etc., the reason why heparin itself inhibited paw edema is that it might have increased the level of some endogenous anti-inflammatory compounds. Karlsson and Marklund [7] reported that EC-SOD B and C can be released from the vascular endothelial cell surface by a relatively high dose of heparin. We reported previously that an appropriate dose of heparin released EC-SODs from vascular endothelial cells and suppressed paw edema induced by either ischemia/reperfusion or carrageenan [9, 10]. If heparin increased the plasma EC-SOD levels, the superoxide-dependent inflammatory process evoked in carrageenan-treated paw could be suppressed by the circulating EC-SODs. It should be noted that the inhibitory effect of heparin alone was more

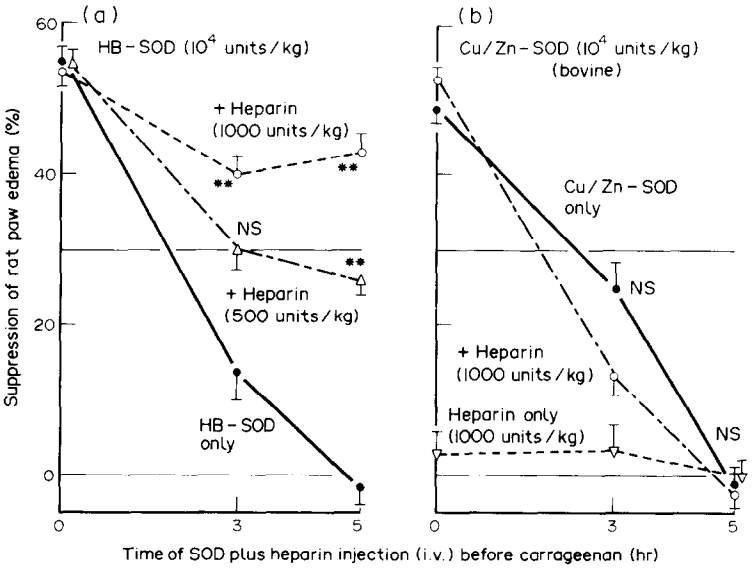

Fig. 5. Prolonged duration of suppressive effect of HB-SOD (a) and no change in effect of bovine Cu/Zn-SOD (b) on rat carrageenan paw edema when heparin was co-injected. SOD and heparin mixture was injected (i.v.) at indicated time before carrageenan injection. Suppression (%) by HB-SOD + heparin was statistically different from that by heparin alone (** $P < 0.01$; NS, not significant). No suppression by 1000 units/kg heparin alone was observed (b). This curve can also be inserted directly in (a). Some variation exists and 1000 units/kg heparin showed 3% suppression instead of 10% as seen in Fig. 4.

marked in mice than rats (see Figs 2 and 4). The fact that EC-SOD C is present in mice but not in rats [7] could explain the greater suppression by heparin alone in mice (Fig. 1). Less suppression by heparin in rats may be due to the release of weak heparin-binding EC-SOD B. HB-SOD and heparin showed additive suppression both in mice and rats. In contrast, the addition of heparin decreased the suppression by SOD in mice and rats (Fig. 2). It should be noted also that the suppressive effect of HB-SOD in mice was a little less with 2000 units/kg of heparin than with 1000 units/kg when the suppressive effect of heparin alone was subtracted (Fig. 2). An excess dose of SOD had a lesser anti-inflammatory effect than did an appropriate amount of the enzyme [12]. Since dismutation of superoxide generates toxic hydrogen peroxide, the low-anti-inflammatory action of a high dose of SOD may be due possibly to the rapid formation of this hazardous oxygen species. Hence, the hyperbolic effect of heparin injected with HB-SOD may depend on an excess of SODs (heparin released EC-SOD plus HB-SOD) in the circulation.

There was no difference in suppressive effect on rat carrageenan-induced paw edema between HB-SOD and SOD when ED_{30} values were compared (Fig. 1), yet only HB-SOD held the capacity to suppress edema for up to 3 or 5 hr after being administered with heparin (Fig. 5). One possible reason for this is the stability of the HB-SOD–heparin complex in the blood stream which enables it to scavenge O_2^- for a long time near or on the blood vessel surface. In our preliminary experiments, injected HB-SOD bound principally to the liver and kidney of rat from where it was released rapidly into the blood stream by heparin injection. Heparin is able to prevent the binding of HB-SOD to the vessels of these massive organs and offer more HB-SOD to the inflamed site. HB-SOD–heparin complex might form in HB-SOD and heparin mixture before injection, allowing only a little HB-SOD to bind to the liver and kidney and a large amount of this complex to be maintained in the blood stream. There are two possible mechanisms for the anti-inflammatory action of this complex. One is direct action of heparin-bound HB-SOD in scavenging O_2^- at the inflamed site. If HB-SOD–heparin had no O_2^- scavenging capacity, dissociation releasing unbound HB-SOD could be supposed to occur in the low pH environment of the inflamed site. A continuous low supply of active HB-SOD to target cells from the stable complex might permit a long-lasting effect of this derivative.

It has been reported that endothelial cells have two types of binding site for diamine oxidase which decomposes histamine [15]. Arterial smooth muscle cells also synthesize two types of functionally different heparin-like proteoglycans [16]. Binding of EC-SOD, HB-SOD, diamine oxidase or lipoprotein lipase [17] to heparan sulfates occurs electrostatically. Thus, these enzymes may compete for the limited number of heparin-like binding sites on vascular endothelial cells and the metabolic fate of pro-inflammatory factors (O_2^-, histamine, lipid peroxides etc.) must be modulated in a complex manner by heparin. On the other hand, the degranulation of mast cells can supply endogenous heparin which might modify the localization of EC-SOD, HB-SOD

etc. In any case, Carr [18] reported the anti-inflammatory action of heparin (i.v.) in the dorsal skin of rabbits and we showed the prolonged effect of HB-SOD with heparin in this work.

Molecular mechanisms by which oxidative tissue injury could be reduced more efficiently with HB-SOD–heparin complex should be studied further. Apart from the possible therapeutic applications of HB-SOD, this modified enzyme may be a useful tool in the study of tissue injury in which oxidative stress in and around vascular endothelial cells plays a critical role.

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