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SUPPRESSION OF CARRAGEENAN PAW OEDEMA IN RATS AND MICE BY HEPARIN-INDUCED EC-SODs

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Carrageenan-induced paw edemata of mice and rats were suppressed by 1-4 × 10^3 U/kg intravenous injection of heparin. High doses were less suppressive, corresponding well to the increase in plasma SOD activity. This biphasic dose response curve was also observed in our ischemic paw model of mice. Increased SOD appeared as high molecular EC-SOD C (in mice) and B (in rats) as a result of its sensitivity to a copper chelator and long retention time in the blood stream, compared to the short life of cytosolic Cu, Zn-SOD. EC-SOD C (135 kDa) failed to be detected in the plasma of heparin-injected mice by way of nitroblue tetrazolium staining after PAGE electrophoresis. Instead, SOD activity was found near 270 kDa. An excess heparin-loaded subunit of this enzyme might become inactivated or might not be able to fix to a pocket where EC-SOD eliminates O_2^-, to protect the endothelium, Electrophoresis dissociates the excess heparin resulting in an active form of the enzyme. Paw edemata of rats were less sensitive because this species lacks the strongly heparin-binding EC-SOD C and has only the weakly heparin-binding EC-SOD B, Ischemia-induced mitochondrial swelling of the paw muscle was observed by electron microscopy and was prevented by heparin injection.

KEY WORDS: Heparin, EC-SOD, ischemic paw edema, carrageenan paw edema, endothelium, vascular permeability.

INTRODUCTION

Heparin is known as a potent inducer of many proteins from blood vessels, as well as a typical anticoagulant. Heparin and other sulphated polysaccharides which have no anti-coagulant activity, release lipoprotein lipase[1] and diamine oxidase.[2] Serum ascorbate, α-tocopherol, urate, bilirubin and ceruloplasmin may protect blood vessels, but Marklund has claimed that extracellular superoxide dismutase (EC-SOD) is mainly responsible for the protection of blood vessels. Cytoplasmic Cu,Zn-SOD (32 kDa) and mitochondrial Mn-SOD (45 or 90 kDa) can be detected in serum, but they are believed to be too weak and less adhesive to protect the tissues. Among EC-SODs, there are 3 types, namely non heparin binding A, weakly heparin binding B and strongly heparin binding C. Injected heparin rapidly removes EC-SOD C which is bound on the surface of endothelial cells.[4] Karlsson *et al.*[5] tested only 2,000 U/kg heparin to verify the increase of plasma SOD activity, but we examined various heparin doses and have found a biphasic dose response curve to the increase of plasma SOD activity. Carrageenan and ischemic paw edemata were suppressed in the same manner. Many anti-inflammatory effects of heparin such as Arthus reaction[6] and allograft rejection[7] can possibly be interpreted by the EC-SOD C releasing capacity of heparin.

MATERIALS AND METHODS

Plasma SOD assay

Blood from mice (about 0.5 ml) was added to a 10 ml plastic tube containing 2.0 ml of 0.25 M sucrose and 10 U/ml heparin. Plasma was obtained by centrifugation (2,000 rpm., 15 min., 0–4°C) and hemolized samples were discarded. Five different amounts of aliquot (25–300 μl) of plasma were transferred to the test tube. Plasma SOD activity was determined by our nitrite methods.[8,9]

Assay of ischemic paw edema

Saline or the drug solution (0.5 ml) was injected intravenously (i.v.) in mice by the tail and the right hind leg was bound 14 times with a commercial rubber ring (1 × 1mm, d = 42 mm) at just above the articulation.[10] Bound rubber was scissored off after 20 min. The swollen right and non-bound left paws were measured at 20 min. after natural reflow of blood. Suppression was determined by the gain of paw thickness or weight (4–6 mice) against that of the control. Double rubber rings were used for the rats by binding 12 times. An ischemia (45 min.) and reflow (30 min.) system was adopted in order to know the gain of paw thickness or weight.

Assay of carrageenan paw edema

One percent of carrageenan solution in saline was injected into the foot pad of mice (35 μl) and of rats (100 μl). After determining the curve of paw swelling for 24 hrs, the effect of the drug was evaluated by measuring the paw swelling at 4 hr.

Animals and chemicals

Male ddY (27–33 g) mice and male Sprague-Dawley rats (230–270 g) were obtained from Sizuoka Agr. Coop. Assoc. Heparin sodium (178.9 U/mg, porcine intestinal mucosa), chondroitin sulfate A sodium (bovine trachea) and chondroitin C sodium (shark cartilage) were purchased from Nakarai Chem. Co. (Kyoto). Carrageenan (type IV), Cu,Zn-SOD (bovine erythrocyte, 3,000 U/mg) were the products of Sigma Chem. Co. (St. Louis). Milk xanthine oxidase (XOD, 20 U/ml suspension) was from Boehringer Mannheim GmbH (West Germany). All other chemicals were of analytical grade and obtained from the usual commercial sources.

RESULTS

Increase of plasma SOD activity by heparin

A very rapid augmentation of plasma SOD activity was verified by 2,000 U/kg heparin (i.v.) at 5 min. after heparin injection. A SOD activity of 74 ± 5 nitrite unit (NU)/ml plasma was obtained. This value was 3.5 times that in normal mice (21 ± 2 NU/ml). A gradual decrease in SOD activity (64 ± 4, 54 ± 3 and 18 ± 2 NU/ml at 20, 60 and 120 min. after injection) demonstrated that this increased SOD was not due to cytoplasmic Cu,Zn-SOD for which the half life in the blood stream ($t_{1/2}$) has been reported as 4–6 min. Two thousand U/kg of heparin which was

the only dose examined by Marklund's group,[5] prove in our test to be the optimum dose for increasing plasma SOD (62 ± 4 NU/ml) when determined at 20 min. after heparin injection. One, eight and twenty thousand U/kg heparin resulted 39 ± 3, 39 ± 5 and 28 ± 6 NU/ml, respectively.[9]

Ischemic paw edema of mice

Ischemic paw edema of mice was most potently suppressed by 2,000 U/kg heparin and to a less extent at the higher doses.[9] This suppressive curve paralleled that of plasma SOD activity induced by heparin. Paw swellings of mice which received 1,000, 2,000, 4,000 and 8,000 U/kg heparin, were measured until 120 min. when only a slight degree of suppression could be observed. The most suppressive dose was 2,000 and 4,000 U/kg at any of these observation times. Co-injection of the copper-chelator, sodium diethyldithiocarbamate (DDC-Na, 0.2 g/kg) blocked the increase of plasma SOD suggesting that this SOD was a copper containing one. Combined dosing of catalase shifted the suppressive peak to the lower dose the reason for which was not clear. Suppression of this paw edema decreased gradually when onset of ischemia was retarded after the heparin injection (Figure 1) and in sharp contrast with rapid

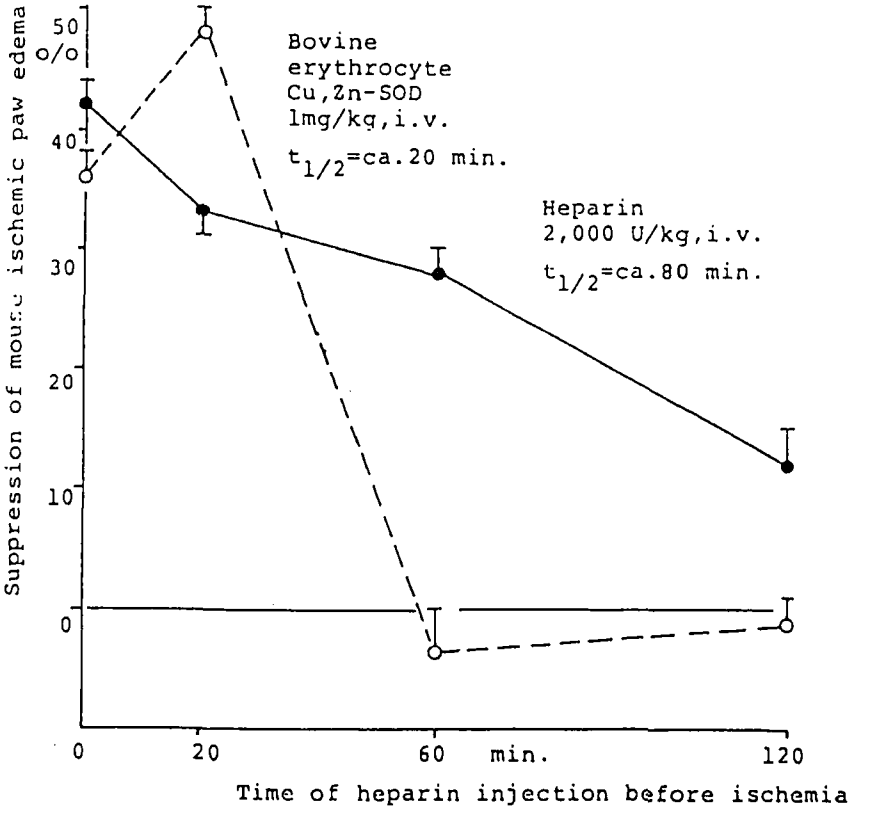

FIGURE 1 Duration of ischemic paw edema suppressive effect of heparin (2,000 U/kg, i.v.) or Cu,Zn-SOD (1 mg/kg, i.v.). Horizontal bars show the S.E. (standard error) of mean of 9 mice.

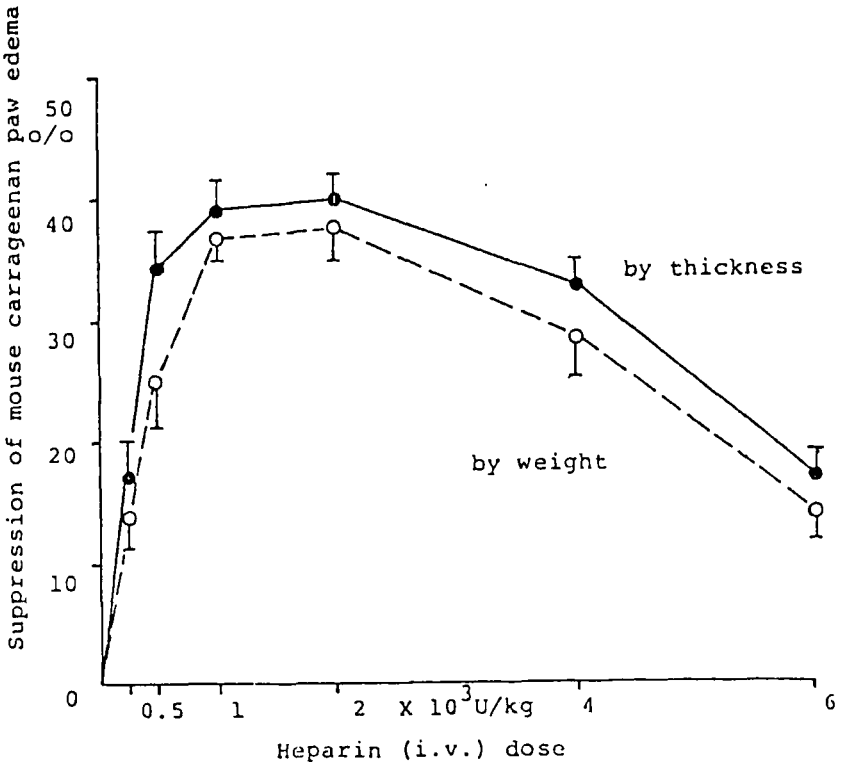

FIGURE 2 Suppression of carrageenan paw edema of mice by heparin. Indicated heparin dose was injected (i.v.) just before the carrageenan and the paw thickness and weight was determined after 4 hrs. Horizontal bars show the S.E. of mean of 9 mice.

decrease of low molecular weight cytoplasmic Cu,Zn-SOD after its injection (no suppression after 1 hr, Figure 1).

Carrageenan paw edema of mice

Carrageenan-induced paw edema of mice was also suppressed by heparin (i.v.) when injected just before the carrageenan and measured at 4 hr (peak of swelling). Maximum suppression (40 ± 2%, $n = 9$) was by 2,000 U/kg (Figure 2). Again, the higher doses were less suppressive for this edema. As 8,000 U/kg heparin was lethal to half of carrageenan-injected mice, so that the heparin dose was limited to 6,000 U/kg. Heparin (2,000 U/kg) resulted in about 30% suppression when injected 1 hr before the carrageenan challenge.

Ischemic and carrageenan paw edema of rats

Maximum suppression of ischemic paw edema of rats was 15 ± 5% by 4,000 U/kg heparin (Figure 3). This suppression may be due to EC-SOD B which binds weakly with heparin, because rats lack EC-SOD C.[5] A little more suppression (23 ± 3%) was observed in carrageenan paw edema at a dose of 2,000 U/kg heparin.

Electron microscopic observation

Three ischemia (20 min.)-reflow (20 min.) treated mice were sacrificed to observe the morphological changes of the paw muscles. They had 20–30% swollen mitochondria. Vacuolization of sarcoplasmic reticulum occurred nearly to the same extent. Muscle fiber bundles was normal and neither invasion nor attachment of leukocytes in blood vessels was observed. Three heparin (2,000 U/kg) received mice showed less mitochondrial and reticulum damages (less than 10%) as in the case of cytosolic Cu,Zn-SOD (1 mg/kg) injected mice. More samples need to be examined to demonstrate the statistical differences.

Polyacrylamide gel electrophoresis (PAGE)

Plasma proteins obtained at 20 min after drug injection (i.v.) were separated on a gel. Markers were phosphorylase b (94 kDa), ferritin (220 kDa) and thyroglobulin (320 kDa). Protein staining showed almost the same pattern in plasma from bovine Cu,Zn-SOD (1 mg/kg), 2×10^3, 8×10^3 and 20×10^3 U/kg heparin. SOD activity staining by nitroblue tetrazolium (NBT) showed a band at about 270 kDa only in plasma from heparin-treated mice. SOD activities of these bands were the same, in contrast to the large difference of total SOD activity of the heparin-treated mouse plasma samples when assayed with the nitrite method before electrophoresis. In direct assay without electrophoresis, plasma from 2×10^3 U/kg heparin injected mice was 61 ± 5 NU/ml compared to 39 ± 4 and 25 ± 6 NU/ml of plasma from 8×10^3 U/kg and 20×10^3 U/kg heparin injected mice. Unclear SOD-like bands (50–100 kDa) were in all samples which might be derived from various proteins, but the expected native EC-SOD C band of the tetramer (135 kDa) was never detected. Excess heparin might be dissociated from inactivated enzyme to make it active.

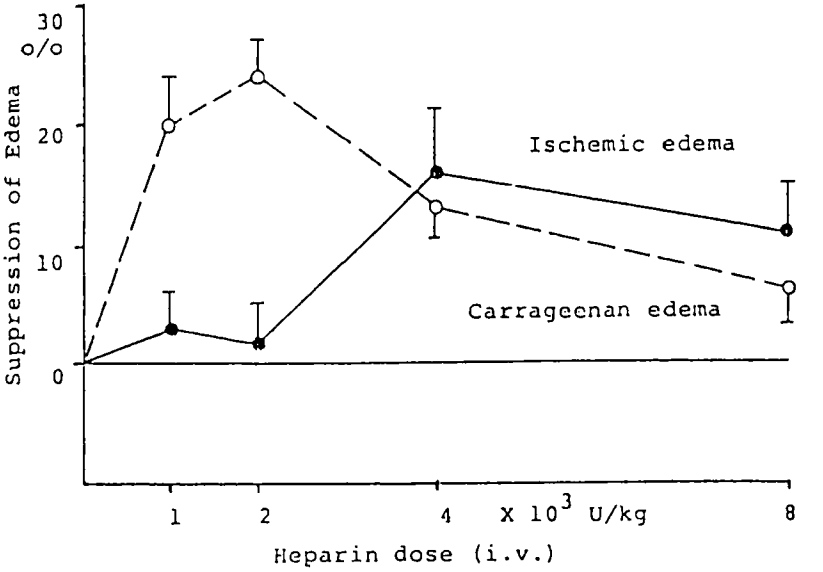

FIGURE 3 Suppression of ischemic- and carrageenan-induced paw edemata of rats. Heparin was injected at the indicated dose just before ischemia onset or carrageenan injection. Edemata were determined as described in methods. Horizontal bars show the S.E. of mean of 5 rats.

DISCUSSION

No significant inhibition of heparin was noticed *in vitro* up to 10^4 U (5.5 mg)/ml in our nitrite method. Luminol-dependent chemiluminescence by neutrophils was reported to be suppressed *in vitro* by heparin or dextrane sulfate,[12] and it has been proposed that the anti-inflammatory effect of heparin is due to the decreased active oxygen production. Nevertheless, we believe this participation is minor, because heparin was needed in high concentration (IC_{50} = 5 mg/ml) and the swelling in our ischemic model was prompt (20 min) not enough to attribute for the attracted neutrophils.

All mammals except rats, have EC-SOD C in their blood vessels, so that intravenous heparin injection resulted in a several fold increase in the plasma EC-SOD C level.[5] We verified this fact and found the simultaneous suppression of ischemic paw edema of mice. This increase of SOD activity may explain, at least in part, the beneficial effect of heparin reported in anaphylactic shock[13] or immune nephritis of rabbits.[14] Ameriolation of human rapidly progressive glomerulonephritis by heparin was observed without any change of glomeruli fibrinogen deposition.[15]

Rats lack the strong heparin-binding EC-SOD C[5] and this may explain our data that the suppression of both ischemic and carrageenan induced paw edemata of rats were moderate (max. 23%). There are reports that heparin was effective to prevent the inflammatory damage to rats. Rammer[16] suppressed the postichemic renal damage of rats by 2,000 U/kg heparin (i.v., 5 min before). Rosenman *et al.*[17] arrested the development of rat nephritis induced by the anti-kidney serum. Rats have no EC-SOD C, but possess a large amount of weak-heparin binding EC-SOD B (dimer) in plasma. In these models, it is possible that heparin serves to transport EC-SOD B efficiently into the kidney. The optimum suppression of all animal models that we tested, was attained in the range of 1–4 × 10^3 U/kg. The reason why higher doses of heparin showed less suppression has yet to be clarified. As plasma SOD activity was also lower with the high heparin doses, so one possibility was the lower release of EC-SOD C from the endothelium. However, our preliminary examination by PAGE electrophoresis excluded this possibility. During electrophoresis heparin might be dissociated from excessively heparin loaded EC-SOD C (inactive state) making this enzyme active. Heparin bound EC-SOD C has been reported as about 270 kDa by Adachi *et al.*[18] as we detected this time. They supposed that heparin bound EC-SOD C became bulky to show the apparent high molecular weight.

Another possibility that high dose heparin resulted in less SOD activity in plasma, was the inactivation of EC-SOD C by excessively loaded heparin. This enzyme is a tetramer and has 4 catalytic sites and 4 heparin binding positively charged C-terminals (3 lysine and 6 arginine residues in 20 amino acids).[18] An appropriate amount of heparin can occupy the C-terminals of 1–3 subunits and may inactivate the catalytic sites of these subunits perhaps by conformational change. None the less, SOD activity must be kept by the remaining 3–1 subunits. When all subunits are occupied by heparin, there might be no SOD activity. It is not yet proved whether heparin binding to the basic amino acids inhibits the activity of EC-SOD C or not, but the modification of only one lysine or histidine significantly decreased the activity of cytosolic Cu,Zn-SOD.[19,20] The amino acid sequence and conformation around the catalytic site in cytosolic- and EC-SOD is very similar. Following on this our hypothesis, EC-SOD C in the normal state, which is bound weakly to the sulphated glycosaminoglycans of endothelium, is possibly inactive. Exogeneously injected EC-SOD C (135 kDa) was

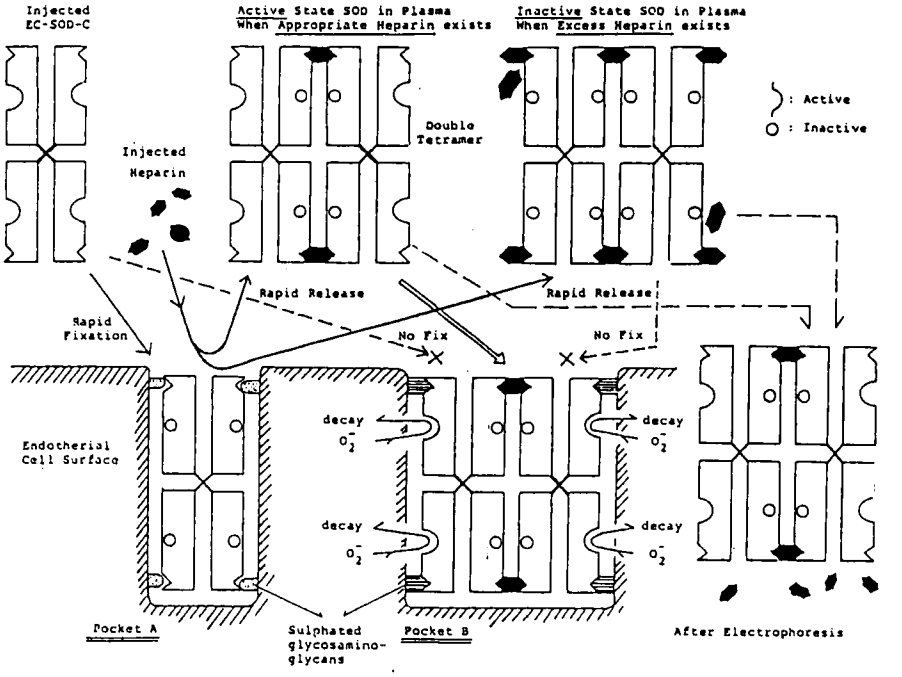

FIGURE 4 Hypothetical mechanism of EC-SOD C protective actions. EC-SOD C may be attached weakly in pocket A as inactive single tetramer in normal state. Appropriate amount of heparin splits off the enzyme by forming enzymatically active EC-SOD C in plasma. This double tetramer may fit for pocket B where it can eliminate O_2^- and protect endothelium. Excess heparin may result the inactivation of released EC-SOD C by blocking the catalytic site and hamper the attachment to pocket B. Electrophoresis detaches only excess heparin and maintains the enzyme as SOD active double tetramer. Injected EC-SOD C might once attached to pocket A and shows it biological effects after transfered to pocket B by the bridge-formation with appropriate amount of heparin or natural heparin-like carrier.

trapped rapidly to the surface of endothelial cells which reappeared by heparin injection according to Karlsson *et al.*[4]

It is important whether EC-SOD C alone can suppress the inflammatory changes or heparin co-injection is essential. The authors prefer the idea that heparin facilitates the transport of EC-SOD C or B to the inflamed site. We supposed the existence of two different sites of EC-SOD C binding on endothelial cell surface (Figure 4). Pocket A fixes the natural state of EC-SOD C as a tetramer and renders this enzyme to the inactive state. Pocket B fits specifically for the double tetramer enzyme bridged by heparin or heparin-like molecule. This type of bound EC-SOD C functions as O_2^- dismutating catalyst and suppress the vascular permeability increase. Very little amount of heparin is known to make the apparently large molecular size of EC-SOD C (about 270 kDa),[18] so heparin works at first to form a double tetramer. Excess heparin may attach to the C-terminals of the resting subunits resulting in no O_2^- scavenging capacity due to the lack of EC-SOD C attachment to the pocket B. This pocket can be supposed to be more exposed in the damaged part of endothelium. The inflamed region is believed to be acidic and is possible to facilitate the fixation of heparin-bridged EC-SOD C to pocket B. The condition of PAGE electrophoresis is

very likely to dissociate only excessively bound heparin and heparin-bridged enzyme appeared as SOD active band of about 270 kDa.

Heparin and protein binding can be influenced by divalent cations (Ca^{+2} and Mn^{+2}) according to Monge *et al.*[21] Delicate regulation of important protein release into plasma is possibly controlled by these cations *in vivo*. Human neutrophil elastase inactivates antithrombin which can be promoted by heparin[22] suggesting the possible autoregulatory effect of heparin anti-coagulant activity. There are many effects of heparin for which the mechanism has not been clarified. Non anti-coagulant β-cyclodextrine tetradecasulfate suppressed 100 times more effectively the tumor angiogenesis than heparin when co-injected with steroid.[23] Potentiation of fibroblast growth factor (FGF) by heparin[24] is another topic up-to-date. Intermediation of EC-SOD C is worth of consideration to investigate the mechanism of these heparin actions. Heparin and EC-SOD C conjugates in plasma have longer durations of suppressive effects on mouse ischemic paw edema than free cytosolic Cu,Zn-SOD (Figure 1). This corresponds well to the decline of SOD activity in plasma. Our preliminary electron microscopic observation demonstrated the mitochondrial swelling of paw muscle tissue obtained from ischemia-reflow submitted mice. This ischemic paw edema of mice was suppressed about 50% by 10 mg/kg allopurinol,[10] so the initial source of O_2^- must be xanthine oxidase, but many deformed mitochondria might be the secondary source of O_2^- from their disturbed electron transport system.

In conclusion, EC-SOD C or B might bind to the appropriate amount of heparin or heparin-like substances to be transferred and fixed on endothelium. Another typical such example is diamine oxidase (DAO) which is reported[25] to have at least two binding receptors on endothelium. It is very likely that EC-SODs bind to different pocket as supposed in this paper and one is for natural single EC-SOD C or B and the other may be for heparin-bridged double tetramer EC-SODs. The bound EC-SODs in the later manner could eliminate O_2^- on the endothelium and protect it from oxidant damage. If the leukocytes lack the pocket B, EC-SOD C cannot attach to it and there may be no blocking effect on the active oxygen derived bactericidal activity of leukocytes.

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Addendum: A new report appeared to show a large release of vascular heparan sulfate into perfusate (little in urine) after the ischemia of rat kidney (P. Vassiliou et al.: Biochem. Int'l., *19*, 1241, 1989). This component is very possible to be a natural carrier of EC-SODs to make minimum the oxidant damage.