# Binding of human xanthine oxidase to sulphated glycosaminoglycans on the endothelial-cell surface

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Much evidence has suggested that the superoxide generated by xanthine oxidase (XOD) within the endothelial cell triggers characteristic free-radical-mediated tissue injuries. Although it has been reported that XOD exists not only in the cytoplasm, but also on the outside surface of the endothelial cell membrane, it is not clear how XOD localizes on the outside of the plasma membrane. Purified human xanthine oxidase (h-XOD) had an affinity for heparin–Sepharose. The binding was largely independent of the pH over the physiological range, whereas it tended to increase at lower pH and to decrease at higher pH. Exposure of h-XOD to the lysine-specific reagent trinitrobenzenesulphonic acid or the arginine-specific reagent phenylglyoxal caused it to lose its affinity for heparin–Sepharose. The binding of h-XOD to heparin is apparently of electrostatic nature, and both lysine and arginine residues are involved in the binding. h-XOD was found to bind to cultured porcine aortic endothelial cells, and this binding was inhibited by the addition of heparin or pretreatment of the cells with heparinase and/or heparitinase. Intravenous injection of heparin into two healthy persons led to a prompt increase in plasma h-XOD concentration. These results suggest that XOD localizes on the outside surface of endothelial cells by association with polysaccharide chains of heparin-like proteoglycans on the endothelial-cell membranes. Superoxide extracellularly generated by XOD may injure the source-endothelial-cell membrane and also attract and activate closely appositional neutrophils, which themselves actually cause progressive oxidative damage.

## INTRODUCTION

It has been suggested that active oxygen species derived from molecular oxygen contribute to tissue injury. Endothelial cells have been proposed as the initial site of tissue injury by the active oxygen species in the vascular system, because they are ubiquitous and located at the blood/tissue barrier [1]. Moreover, extracellular fluids outside of endothelial cells contain only small quantities of endogenous enzymes, such as superoxide dismutase, catalase and glutathione peroxidase [2,3], protective against the active oxygen species. It appears that vascular endothelial cells intrinsically generate active oxygen species when subjected to anoxia and reoxygenation, conditions observed in ischaemic and reperfused tissues [4].

Endothelial cells are a rich source of the superoxide-generating enzyme xanthine oxidase (XOD) [1]. XOD has been demonstrated to be the source of most of the observed active oxygen species, because of the marked inhibition of radical generation by the potent XOD inhibitors allopurinol and oxypurinol [4,5]. XOD catalyses the two-step oxidation of purine, such as hypoxanthine, through xanthine, to urate. *In vivo*, the enzyme may exist in either of two forms, each of which catalyses the above reaction: the dehydrogenase form, which uses $NAD^+$ as an electron acceptor and therefore does not generate superoxide, and an oxidase form, which uses molecular oxygen as an electron acceptor and thereby produces the superoxide. The dehydrogenase form, which predominates in most tissues under normal conditions, can be converted into the oxidase form by irreversible limited proteolysis and/or reversible oxidation of thiol groups in ischaemia [6,7].

In a number of tissues, including mammary gland, liver, heart, lung and intestine, XOD has been found in endothelial cells of capillaries by immunohistochemical research [1,8]. Recently, it has been demonstrated that XOD is not only a cytoplasmic enzyme, but that it is also localized on the outside surface of the endothelial-cell plasma membrane [9]. However, it is still not known how XOD localizes on the cell surface.

In the present study the binding of XOD to heparin–Sepharose and to cultured endothelial-cell surfaces via heparan sulphate proteoglycans are explored. The effect of heparin injection on the plasma XOD level in man has also been studied.

## EXPERIMENTAL

### Materials

Xanthine, horseradish peroxidase (HRP, type IV), protamine and heparinase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phenylmethanesulphonyl fluoride (PMSF), aprotinin, Nitroblue Tetrazolium (NBT) and trinitrobenzenesulphonic acid (TNBS) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phenylglyoxal was purchased from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). Heparin was obtained from Kodama Co. (Tokyo, Japan). DEAE-cellulose, hydroxyapatite and heparitinase were purchased from Seikagaku Co. (Tokyo, Japan). DEAE-Sepharose, Sephacryl S-300 and heparin–Sepharose CL-6B were products of Pharmacia LKB Biotechnology (Uppsala, Sweden). 96-Well immunoplates were purchased from Nunc (Roskilde, Denmark). Dulbecco's modified Eagle medium (DMEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Porcine aortic endothelial (PAE) cells were kindly given by Takeda Chemical Industries (Osaka, Japan).

Abbreviations used: h-XOD, human xanthine oxidase; HRP, horseradish peroxidase; PAE cell, porcine aortic endothelial cell; PMSF, phenylmethanesulphonyl fluoride; NBT, Nitroblue Tetrazolium; TNBS, trinitrobenzenesulphonic acid; DMEM, Dulbecco's modified Eagle medium; EDRF, endothelium-derived relaxing factor; EC-SOD, extracellular-superoxide dismutase.

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### Purification of human milk xanthine oxidase (h-XOD)

h-XOD was purified from collected human milk by the method described by Zeise and Zikakis [10], but with several modifications.

Sodium salicylate and $Na_2EDTA$ were added into the thawed milk to final concentrations of 2 mM (salicylate) and 0.01% ($Na_2EDTA$). A 600 ml portion of the mixture was added to an equal volume of 0.2 M $K_2HPO_4$ containing 8 mM sodium salicylate, 2 mM cysteine, 0.5 mM PMSF and 100000 units of aprotinin, and the resultant mixture was then incubated at 40 °C for 2 h with frequent stirring. After the sample was cooled to 4 °C, Triton X-100 was added to a final concentration of 1% (v/v) and the mixture was stirred for 15 min. All subsequent procedures were carried out at 4 °C. Solid $(NH_4)_2SO_4$ was added to a concentration of 25% saturation, and the mixture was centrifuged at 10000 ***g*** for 30 min. The upper fat layer was discarded, and the liquid layer was filtered through glass wool. $(NH_4)_2SO_4$ (final concn. 40% satn.) was added to the filtrate. After stirring overnight, the solution was centrifuged at 10000 ***g*** for 30 min. The precipitate was dissolved with a minimal volume of distilled water and dialysed against 5 mM sodium pyrophosphate buffer, pH 8.5 containing 2 mM sodium salicylate and 0.005% $Na_2EDTA$ (Buffer A). The dialysed enzyme was passed through a column (3 cm × 25 cm) of DEAE-cellulose equilibrated with Buffer A. The active fractions were pooled and concentrated with YM-10 membrane filter, followed by application to a column (4.5 cm × 10 cm) of DEAE-Sepharose equilibrated with Buffer A. The column was eluted with a linear gradient of sodium pyrophosphate buffer, pH 8.5, between 5 mM and 0.1 M, containing sodium salicylate and $Na_2EDTA$ at the same concentrations as in Buffer A. The active fractions were pooled, concentrated with a YM-10 membrane filter and dialysed against Buffer A. The enzyme was then applied to a column (1.2 cm × 10 cm) of hydroxyapatite equilibrated with Buffer A. XOD was eluted stepwise from the column by successive washing with Buffer A containing 0.1, 0.2, 0.3, 0.4 and 1 M NaCl. XOD was eluted mainly in the 0.3 and 0.4 M NaCl steps. The concentrated XOD preparation was applied to a Sephacryl S-300 column (2 cm × 90 cm) equilibrated with Buffer A, and active fractions were pooled and concentrated with a YM-10 membrane filter.

The $A_{280}/A_{450}$ ratio of the purified enzyme was about 5.2. The purity of h-XOD was checked by SDS/PAGE (7.5% polyacrylamide gel) under reducing conditions. A single protein band with a molecular mass of 145 kDa was observed upon staining of the gel with Coomassie Brilliant Blue R250. The $A_{280}/A_{450}$ ratio and monomeric molecular mass are almost identical with those reported for XODs from other sources [11,12].

### Assay of h-XOD activity

Enzyme solution (20–50 $\mu$l) was added to 1 ml of 50 mM sodium carbonate buffer, pH 10.2, containing 0.12 mM xanthine, 0.12 mM $Na_2EDTA$, 60 $\mu$g/ml BSA and 0.03 mM NBT, and then incubated at 37 °C for 30 min. The enzyme reaction was stopped by the addition of 50 $\mu$l of 6 mM $CuCl_2$ solution, and the absorbance at 560 nm was measured.

### E.l.i.s.a. for h-XOD

The antisera to h-XOD were raised in Japanese White rabbits by subcutaneous injection of the purified h-XOD (200 $\mu$g/rabbit) in Freund's complete adjuvant. The same immunization was repeated four times every 2 weeks, and the rabbits were bled 1 week after the final injection. The antibody was purified by $(NH_4)_2SO_4$ precipitation (33% satn.) followed by the DEAE-cellulose column chromatography.

The HRP-labelled antibody was prepared with glutaraldehyde as a cross-linking reagent by the method as described previously [13].

An 80 $\mu$l portion of 50 $\mu$g/ml antibody dissolved in 50 mM sodium carbonate buffer, pH 9.5, containing 0.02% $NaN_3$ was added into each well of Immunoplates and left overnight at 4 °C. Each well was washed with 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.05% Tween 20 and 0.02% merthiolate (washing buffer). The remaining protein-binding site was blocked with 300 $\mu$l of 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1% BSA, 0.05% Tween 20 and 0.02% merthiolate (blocking buffer). The plate was then left to stand at 4 °C until use.

Portions (70 $\mu$l) of sample or standard diluted with blocking buffer were added to the wells. The plate was incubated for 2 h at room temperature and washed three times with the washing buffer. Then 80 $\mu$l of HRP-labelled antibody diluted with blocking buffer was added to each well, and the plate was incubated for 2 h at room temperature, followed by washing three times with washing buffer. Substrate solution (150 $\mu$l of 0.1 M McIlvain buffer, pH 6.5, containing 3 mg/ml *o*-phenylenediamine and 0.2 mg/ml $H_2O_2$) was then added to each well and the plate was incubated for 30 min at room temperature. The enzyme reaction was stopped by the addition of 50 $\mu$l of 2.25 M-$H_2SO_4$, and the absorbance at 492 nm was measured.

### Heparin–Sepharose column chromatography

The samples were dialysed against 25 mM sodium phosphate buffer, pH 7.5, or mixed with excessive volume of 25 mM buffer at various pH values (sodium acetate buffer, pH 5.5, sodium phosphate buffer, pH 6.5 and 7.5, Tris/HCl buffer, pH 8.5 and 9.5) before application.

The samples were applied to a heparin–Sepharose column equilibrated with the indicated buffer and washed with the same buffer. The bound h-XOD was then eluted with stepwise additions of buffer containing increasing concentrations of NaCl (0.1 M increments) or a linear gradient of NaCl (0 → 1 M) in the buffer.

### Binding of h-XOD to PAE cells

The cells were cultured and maintained in DMEM supplemented with 10% fetal-calf serum. They were kept in a 5%-$CO_2$ atmosphere at 37 °C.

The experiments were performed using confluent endothelial cell monolayers in 35 mm-diameter tissue-culture dishes as follows. The monolayers were rinsed three times with 1 ml of DMEM containing 10 mM Hepes and 0.5% BSA (DMEM-BSA), 1 ml of DMEM/BSA with or without 1 unit of heparitinase and/or 2 units of heparinase were then added and the cells were incubated for another 2 h in a $CO_2$ incubator. After washing three times with 3 ml of DMEM/BSA, 13 $\mu$g of h-XOD in 0.5 ml of DMEM/BSA was added and the cells were incubated at 4 °C for 2 h. At the end of the incubation period, the unbound h-XOD was removed and the cells were washed three times with 1 ml of PBS. The cells were then incubated for 10 min at 4 °C with 0.5 ml of PBS containing 10 mg/ml heparin to release h-XOD from the cell surface. After washing cells three times with 1 ml of PBS, cells were detached with a rubber policeman and sonicated in 1 ml of PBS.

### Plasma sampling

Blood samples were collected into siliconized glass tube containing CPD solution (0.1 M sodium citrate/17 mM citric acid/0.129 M glucose/16 mM $NaH_2PO_4$). After centrifugation (1500 *g* for 15 min) the plasma samples were kept at −30 °C until used.

## RESULTS

### Binding of h-XOD to heparin–Sepharose

Figure 1 shows a heparin–Sepharose column chromatography of purified h-XOD. h-XOD had an affinity for heparin and eluted as a single peak having both catalytic and immunological activities with the buffer containing 0.3–0.4 M NaCl.

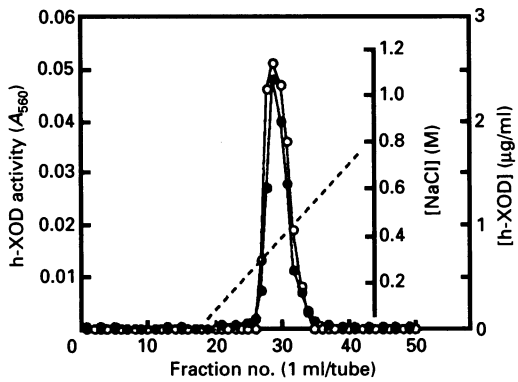


**Figure 1 Heparin–Sepharose column chromatography of purified h-XOD**

The chromatography was carried out at room temperature on a heparin–Sepharose column (volume 2 ml) equilibrated with 25 mM sodium phosphate buffer, pH 7.5. A 100 μl portion of 200 μg/ml purified h-XOD was dialysed against above buffer before application to the column. The sample was applied at 1 ml/min and was eluted by a linear gradient of NaCl in the buffer (0 → 1 M) at 1 ml/min. ○, XOD activity assayed by the method described in the Experimental section; ●, concentration of h-XOD assayed by e.l.i.s.a. ----, concentration of NaCl in buffer.

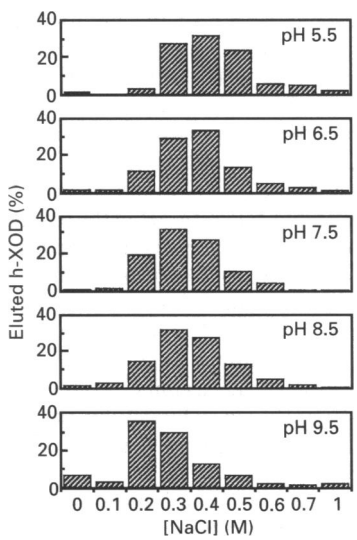


**Figure 2 pH-dependence of binding of h-XOD to heparin–Sepharose**

Portions (10 μl) of purified h-XOD (650 μg/ml) were mixed with 400 μl of various 25 mM buffers (sodium acetate buffer, pH 5.5; sodium phosphate buffer, pH 6.5 and 7.5; Tris/HCl buffer, pH 8.5 and 9.5) and 100 μl of heparin–Sepharose was equilibrated with each buffer and gently shaken at room temperature. After 10 min, the suspension was centrifuged and the supernatant was collected. Bound h-XOD was then eluted from the gel by stepwise addition of buffer containing an increasing concentration of NaCl (0.1 M increments). The h-XOD concentration in the supernatants was assayed by e.l.i.s.a.

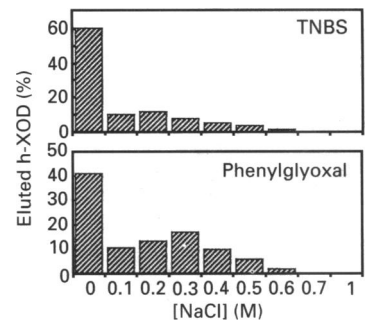


**Figure 3 Effect of TNBS and phenylglyoxal modification on h-XOD**

Purified h-XOD (650 μg/ml) was dialysed against distilled water before use. h-XOD (100 μl) was incubated with 10 mM TNBS or 10 mM phenylglyoxal in 0.2 M $NaHCO_3$ (20 μl) for 2 h at room temperature, followed by application on Sephadex G-25 equilibrated with 25 mM sodium phosphate buffer, pH 7.5. h-XOD fraction was pooled and concentrated with the above buffer saturated with $(NH_4)_2SO_4$. Modified h-XOD sample (480 μl) re-dialysed against 25 mM sodium phosphate buffer, pH 7.5, was mixed with 120 μl of heparin–Sepharose gel equilibrated with the above buffer. The chromatography and the assay of h-XOD concentration were done as described in the legend to Figure 2.

The effect of pH was assessed by determining the releasing effect of stepwise addition of NaCl to h-XOD previously bound to heparin–Sepharose in the different buffers. The heparin affinity of h-XOD did not vary with pH over the range 7.5–8.5. The binding strength tended to increase at lower pH, whereas a significant decrease in heparin affinity was observed at pH 9.5 (Figure 2).

### Effect of protamine and heparin on binding of h-XOD to heparin–Sepharose

Protamine is one of several basic proteins that are made up of many basic amino acids and bind avidly to heparin; it is used clinically as a heparin antidote. Protamine inhibited the binding of h-XOD to heparin–Sepharose (results not shown). This binding was inhibited also by the addition of heparin (results not shown).

### Modification of lysine and arginine residues of h-XOD

The putative involvement of lysine and arginine residues in the binding of h-XOD to heparin–Sepharose were tested by exposing the h-XOD to the lysine-specific reagent TNBS and the arginine-specific reagent phenylglyoxal. The enzymic activity of h-XOD was much inhibited by TNBS (remaining activity was 4.5%), but not by phenylglyoxal (remaining activity was 92.0%). After modification with TNBS there were almost no heparin-affinity forms of the h-XOD left. On the other hand, only roughly half of the h-XOD lost its affinity for heparin–Sepharose (cf. Figure 2, pH 7.5) when arginine residues were modified with phenylglyoxal (Figure 3).

### Binding of h-XOD to PAE cells

The binding of h-XOD to PAE cells was studied by incubating cells with h-XOD at 4 °C for 2 h. The cell surface-bound h-XOD was released by the addition of heparin.

To investigate whether h-XOD bound to cells via surface heparan sulphate proteoglycans, the effect of heparin addition

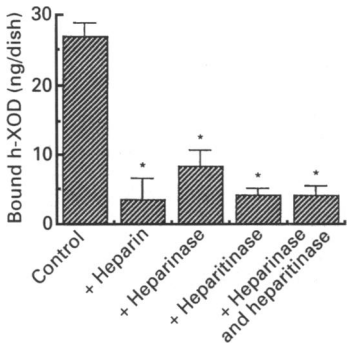


**Figure 4 Binding of h-XOD cultured PAE cells**

The binding of h-XOD to PAE cells was studied as described in the Experimental section. The concentration of h-XOD in each fraction was assayed by e.l.i.s.a. Heparin-added cultures and endoglycosidase-treated cultures were compared with control cultures using Student's *t* test (*$P < 0.001$).

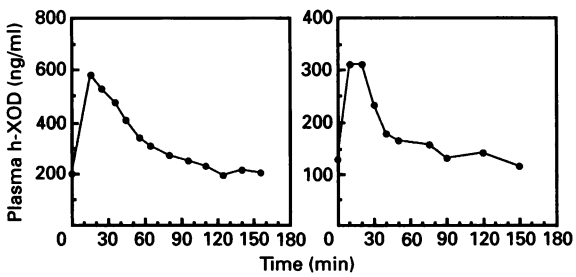


**Figure 5 Effect of intravenous heparin injection on plasma h-XOD**

Heparin (50 i.u./kg body weight) was injected at zero time into two healthy males, and plasma samples were collected before (0), and at indicated times after, injection. The concentration of h-XOD was assayed by the e.l.i.s.a. described in the Experimental section.

was examined. h-XOD was incubated in media containing 1 mg/ml heparin. The percentage of the cell-surface h-XOD was markedly reduced compared with the control test (Figure 4).

To examine whether the removal of heparan sulphate proteoglycans on the endothelial-cell surface affected the binding of h-XOD, the cells were incubated in the presence of heparinase and/or heparitinase for 2 h at 37 °C, then h-XOD was allowed to bind to the cells at 4 °C. Heparinase treatment decreased the binding of h-XOD to the cells by 68% compared with control, and heparitinase treatment more efficiently decreased the binding by 84% (Figure 4).

### Heparin-induced release of h-XOD to human blood plasma

Figure 5 shows that an intravenous injection of 50 i.u. of heparin/kg body weight leads to rapid 2.6–2.9-fold rise in plasma h-XOD levels. The plasma h-XOD level then slowly declined, approaching the initial values after 2 h.

## DISCUSSION

Active oxygen species have been implicated in a number of disparate disease processes. Two possible sources have been identified for the production of active oxygen species: the enzyme XOD [14] and activated neutrophils [15]. Circulating neutrophils have long been known to be an important source of superoxide generation. On the other hand it has been proposed that the superoxide generated by XOD within the endothelial cell causes a free-radical-mediated chain reaction, and the neutrophils may then respond to the initial endothelial-cell injury and/or to the chemotactic properties of the superoxide generated by XOD [5].

Immunohistochemical studies appear to localize XOD in the microvascular endothelium of a number of organs [1,8]. They also showed that XOD was observed through the whole cytoplasm within the endothelial cells [8]. A model has been proposed that superoxide generated by XOD in endothelial cells may attract and activate neutrophils, which themselves actually cause most of the oxidant damage [5]. How this superoxide production by XOD within endothelial cells is communicated to the neutrophils is not yet entirely clear, because superoxide, being a charged molecule generated within the cell, cannot so easily diffuse through the cell membrane [16]. Recently, Bulkley [9] reported that XOD localized not only in the cytoplasm, but also on the outside surface of the endothelial-cell membrane. This would suggest that superoxide generated by XOD on the plasma membrane acts directly as a chemotactic factor, leading to accumulation, adherence and activation of neutrophils. Moreover, plasma-membrane-produced superoxide may play a role as an inactivator of nitric oxide, which is a major form of endothelium-derived relaxing factor (EDRF) [17] and second messenger to increase intracellular free-calcium concentration [18]. However, it is not clear how XOD localizes on the outside surface of endothelial-cell membrane.

The present data show the possibility that XOD localizes on the outside surface of endothelial cells by association with extracellular glycosaminoglycans. Purified h-XOD had moderate affinity for heparin–Sepharose. The binding is apparently of an electrostatic nature, because h-XOD could be displaced by a buffer containing about 0.3 M NaCl. The affinity of h-XOD for heparin might be weaker than that of antithrombin III [19] and extracellular superoxide dismutase C (EC-SOD C) [20]. Since h-XOD bears negative or no net charge at neutral pH (the pI of h-XOD is heterogeneous, ranging from 3.7 to 8.2 [10]), negatively charged sulphate groups of heparin presumably might interact with some clusters of positively charged amino acid residues of this enzyme. At pH 9.5 the affinity of the enzyme for heparin decreased, probably owing to a weak protonation of essential basic amino acid residues. Similar effects of pH on the binding of lipoprotein lipase [21] and EC-SOD C [22] to heparin-substituted gel had been reported. Protamine is a strongly positively charged protein and competed with h-XOD for heparin–Sepharose. These results would suggest the putative involvement of basic amino acid residues in the binding. The studies with the amino-acid specific reagents indicated that both lysine and arginine residues were involved in the binding of heparin. h-XOD comprises 7.8% lysine and 3.4% arginine (of the total amino acid composition) [10]. The heparin-binding sites of EC-SOD C [23,24] and antithrombin III [25] have been identified and appear to involve lysine and arginine residues. However, it is impossible at this point to speculate about the heparin-binding region of h-XOD, since the primary structure of this enzyme has not yet been elucidated.

The experiments presented here demonstrate that h-XOD can bind to cultured endothelial-cell surface. The complete inhibition of binding by the addition of heparin and the failure of the h-XOD to bind to cells pretreated with heparinase and/or heparitinase support the conclusion that h-XOD binds to the polysaccharide chains of heparin-like proteoglycans on the endothelial-cell plasma membrane.

Intravenous injection of heparin led to a prompt increase in plasma h-XOD concentration. It appears that the rise of plasma h-XOD is due to displacement from the heparan sulphate,

located on the endothelial cell surface. Karlsson and Marklund [26] reported that an intravenous injection of 200 i.u. of heparin/kg body weight led to a rapid 4–6-fold rise in plasma EC-SOD C. It was shown that the release of EC-SOD was heparin-dose-dependent, and the percentage of an EC-SOD-releasing-activity of 50 i.u. of heparin per kg body weight was nearly 40% compared with a 200 i.u./kg injection. The ratio of the increase achieved for h-XOD seems to be roughly equal to that for EC-SOD C. The risen plasma h-XOD level slowly declined after a rapid rise, approaching the initial value after 2 h.

None of the results reported here are inconsistent with the notion that XOD localizes on the outside surface of endothelial cells by association with heparan sulphate. XOD-derived superoxide can damage both the source cell and cells in close apposition to endothelial cells, for example, adhered neutrophils. Superoxide generated extracellularly by XOD would initiate a toxic reaction at the membrane. The unsaturated fatty acids present in membrane and transmembrane proteins are susceptible to oxidative damage; increased membrane permeability can be caused by lipid peroxidation, and oxidation of structurally important proteins can cause a breakdown of plasma membrane. One might speculate that cytosolic XOD is released through the damaged plasma membrane. On the other hand, extracellularly generated superoxide may also attract and damage the neutrophils. These neutrophils would bring about the injury of tissues by release of additional active oxygen species, oxidative enzymes such as myeloperoxidase and hydrolytic enzymes.

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