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Heparin-induced release of extracellular superoxide dismutase to human blood plasma

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Extracellular superoxide dismutase (SOD) has previously been shown to be the major SOD isoenzyme in extracellular fluids. Upon chromatography on heparin–Sepharose it was separated into three fractions: A, without affinity; B, with intermediate affinity; and C, with relatively strong heparin affinity. Intravenous injection of heparin leads to a prompt increase in plasma extracellular-superoxide-dismutase (EC-SOD) activity. Heparin induces no release of EC-SOD from blood cells, nor does it activate EC-SOD in plasma, indicating that the source of the released enzyme is the endothelial-cell surfaces. No distinct saturation could be demonstrated in a dose–response curve up to 200 i.u. of heparin per kg body weight, showing that the releasing potency of heparin is lower for EC-SOD than for previously investigated heparin-released factors. Chromatography of human plasma on heparin–Sepharose shows nearly equal amounts of EC-SOD fractions A, B and C. Heparin induces specifically the release of fraction C. The findings point to the existence of an equilibrium of EC-SOD fraction C between the plasma phase and endothelial-cell surfaces. The major part of EC-SOD in the vasculature seems to be located on endothelial-cell surfaces.

INTRODUCTION

Extracellular superoxide dismutase (EC-SOD, EC 1.15.1.1) is the major SOD isoenzyme in extracellular fluids like plasma, lymph and synovial fluid (Marklund *et al.*, 1982, 1986). EC-SOD also occurs in tissues, and in higher concentration (per g wet weight) than in plasma (per ml) (Marklund, 1984*b*). Still, EC-SOD accounts for only a few per cent of the total tissue SOD activity. EC-SOD is a tetrameric, slightly hydrophobic, glycoprotein that contains four Cu atoms and possibly also four Zn atoms (Marklund, 1982). No similarities with other SOD isoenzymes are apparent in amino acid composition (Marklund, 1982) and antigenic properties (Marklund, 1984*a*).

Upon chromatography on heparin–Sepharose, EC-SOD is separated into three fractions: A, without affinity; B, with weak affinity; and C, which is eluted late in a NaCl gradient (Marklund, 1982). Many other proteins with affinity for heparin are known. For some of these, like lipoprotein lipase, hepatic lipase (Krauss *et al.*, 1973), diamine oxidase (Hansson *et al.*, 1966) and platelet factor 4 (Dawes *et al.*, 1978), it has been found that injection of heparin leads to vast increases in the plasma content of these proteins. The release of these factors into plasma is apparently due to displacement from heparin analogues on endothelial-cell surfaces (Busch *et al.*, 1980; Robinson-White *et al.*, 1985). The present paper explores the effect of intravenous heparin injections on the plasma EC-SOD activity in man.

MATERIALS AND METHODS

Pig gut mucosa heparin (sodium salt) was obtained from KABI-Vitrum AB, Stockholm, Sweden. The specific activity was 160 i.u./mg. Heparin–Sepharose and Sephadex G-15 columns (PD-10) were products of Pharmacia Laboratory Separation Division, Uppsala, Sweden.

Handling of blood samples

Blood samples were tapped in Terumo Venoject vacuum tubes with EDTA (38 mmol/litre) as anticoagulant. After centrifugation (2500 *g*, 20 min) the plasma samples were kept at −80 °C until assay.

Analysis of SOD isoenzymes in plasma

SOD was assayed by means of the direct spectrophotometric method employing KO_2 (Marklund, 1976) with modifications as described by Öhman & Marklund (1986). One unit corresponds to 8.3 ng of human Cu,Zn-SOD, 8.8 ng of human EC-SOD and 65 ng of bovine Mn-SOD.

Distinction between isoenzymes in plasma was achieved by means of antibodies towards human Cu,Zn-SOD and EC-SOD immobilized on Sepharose 4B as described previously (Öhman & Marklund, 1986).

The Cu,Zn-SOD activity of the plasma samples was compensated for Cu,Zn-SOD released from erythrocytes lysed during sampling and handling of the blood (Öhman & Marklund, 1986).

Effect on plasma EC-SOD of heparin added to whole blood

Whole blood (20 ml) was tapped from three healthy persons in EDTA-containing tubes. The blood was divided into two equal parts, and to one part was added 30 i.u. of heparin/ml and to the other an equal volume of 0.15 M-NaCl. After incubation for 30 min at room temperature, the samples were centrifuged (2500 *g*, 20 min) and the plasmas collected for SOD analyses.

Abbreviations used: SOD, superoxide dismutase; EC-SOD, extracellular superoxide dismutase.

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Intravenous injection of heparin

Heparin was injected intravenously into two healthy males (aged 34 and 40 years of age) fasted overnight. Blood samples were tapped before heparin injection and at times after as indicated in the text and the Figures.

Chromatography of plasma on heparin–Sepharose

The chromatographies were carried out at room temperature in columns containing 2 ml of heparin–Sepharose with 25 mmol of potassium phosphate/litre, pH 6.5, as eluent. The samples were applied at 4.2 ml/h and when the A_{280} approached baseline, bound components were eluted with a linear gradient of NaCl in the buffer (0–1 mol/litre; total volume 50 ml) at 9 ml/h.

Before application the plasma samples were equilibrated with the elution buffer by means of chromatography on small Sephadex G-15 columns (PD-10). The chromatography resulted in a 3-fold dilution of the samples. The recovery of SOD activity was near 100%.

More than a third of the A_{280} of the plasma binds to heparin–Sepharose under the present conditions (cf. Fig. 3 below) and the procedure was therefore checked for overload and competition of plasma components for the binding of the EC-SOD fractions to the heparin residues. Purified EC-SOD fractions B and C were added to plasma and various amounts of these plasmas chromatographed on the column. More than 4 ml of plasma containing fraction C could be applied without any change in the position of the EC-SOD C peak in the chromatogram and yield of SOD activity. With fraction B, 2 ml of plasma posed no problem, whereas with 4 ml of plasma a slight decrease in the EC-SOD B peak and an apparent increase in the A peak could be seen. The maximal amount of plasma to be applied to the heparin–Sepharose column was therefore chosen to be 2 ml. Addition of 5 i.u. of heparin/ml of plasma (corresponding to the expected plasma concentration after an intravenous injection of 200 i.u. of heparin per kg body weight) had no effect on the elution patterns of fractions B and C.

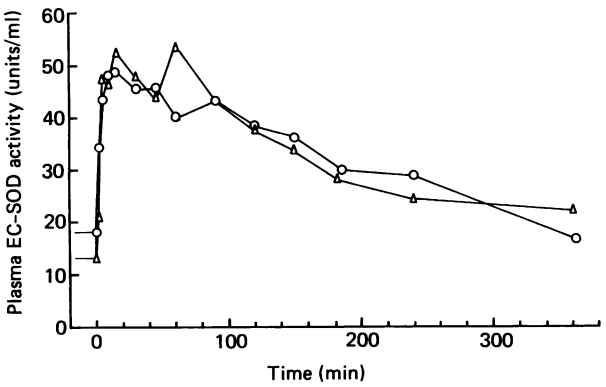

Fig. 1. Effect of intravenous heparin injection on plasma EC-SOD

Heparin (200 i.u. per kg body weight) was injected at zero time into two healthy males (○, △) and plasma collected before and at indicated times thereafter. The EC-SOD activity was determined as described in the Materials and methods section.

RESULTS

Effect of intravenous heparin injection on plasma EC-SOD

Fig. 1 shows that an intravenous injection of 200 i.u. of heparin per kg body weight leads to a rapid 3-fold rise in plasma EC-SOD activity. The maximal increase is approached within 5–10 min. The activity then slowly declines, approaching the initial value after 6 h. There was no effect of intravenous heparin on the plasma Cu,Zn-SOD and cyanide-resistant SOD activities.

Effect of heparin added to blood and plasma on the EC-SOD activity

Addition of heparin to whole blood as described in the Materials and methods section had no effect on the plasma EC-SOD activity. Nor did addition of heparin (5 i.u./ml) directly to plasma result in any change in the EC-SOD activity. The results thus show that the increase in plasma EC-SOD activity as seen in Fig. 1 is not due to release of enzyme from blood cells, nor is it due to activation of the EC-SOD present in plasma.

Dose response of the EC-SOD-releasing activity of intravenous heparin

Fig. 2 shows the dose reponse of intravenous heparin up to 200 i.u./kg body weight on the release of EC-SOD into plasma. Increasing doses of heparin result in increased release of EC-SOD. No plateau is reached, and it is probable that doses over 200 i.u./kg body weight would result in even higher EC-SOD release. Ethical

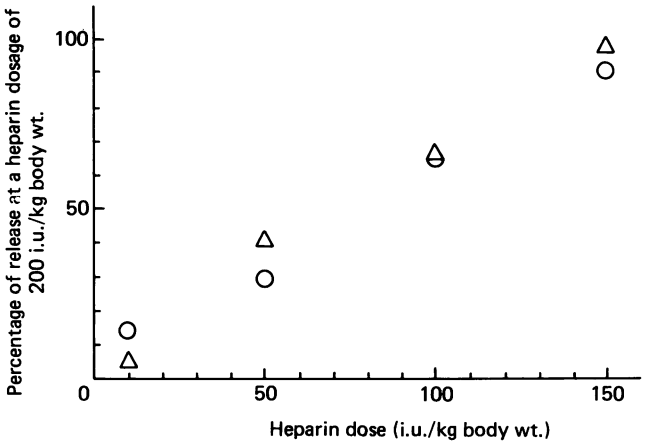

Fig. 2. Dose response of the EC-SOD-releasing activity of intravenous heparin

Heparin at indicated doses was injected intravenously into two healthy males (○, △) and blood collected before and at 10 and 15 min after the heparin injection. At 15 min an additional dose of heparin, to make the total dose 200 i.u./kg body weight, was injected and blood collected 10 min thereafter. EC-SOD was determined in plasma as described in the Materials and methods section. The difference between the pre-heparin EC-SOD activity and the activity after the second heparin injection was taken as 100% release. The differences between the pre-heparin activity and the mean activity of the 10 and 15 min samples after the first dose are presented in relation to the '100% release'. The separate experiments were performed with at least 4-day intervals in between.

Table 1. Separation of plasma EC-SOD into fractions A, B and C

Plasma was chromatographed on heparin–Sepharose as described in the Materials and methods section. EC-SOD fractions A, B and C were pooled as indicated in Fig. 3. The EC-SOD content in pool A was determined as described for plasma in the Materials and methods section. All activity in pools B and C was EC-SOD, since it was adsorbed by anti-(EC-SOD)–Sepharose. The mean yield of EC-SOD activity in the chromatograms was 95%.

Age	Sex	Fraction . . .	SOD activity (units/ml of plasma) A	B	C
41	Female		3.5	5.5	8.7
40	Male		5.9	6.2	7.0
32	Male		2.3	5.2	6.4
33	Female		2.3	5.9	8.3
29	Female		3.3	6.1	7.3
Mean ± S.D. . . .			3.5 ± 1.5	5.8 ± 0.8	7.5 ± 1.0

considerations, however, precluded testing of higher doses.

Analysis of plasma EC-SOD on heparin–Sepharose

Table 1 presents the results of determination of EC-SOD fractions A, B and C in five normal plasma specimens. It is found that the three fractions are approximately equally large in normal plasma. The separation into three fractions is apparently not due to secondary degradation *in vitro*, since the pattern for a plasma specimen was identical before and after storage for 3 days in a refrigerator. Fig. 3 shows the effect of intravenous heparin on the composition of EC-SOD fractions in plasma. It is found that, in the analysed person, heparin leads to a significant increase only in fraction-C activity. Activity in fractions A and B remain essentially unaltered. In a second analysed person (results not shown) the effect of heparin was essentially identical. Fraction-C activity rose from 7 to 32 units/ml of plasma.

DISCUSSION

Intravenous injection of heparin leads to a prompt increase in plasma EC-SOD activity. Heparin does not

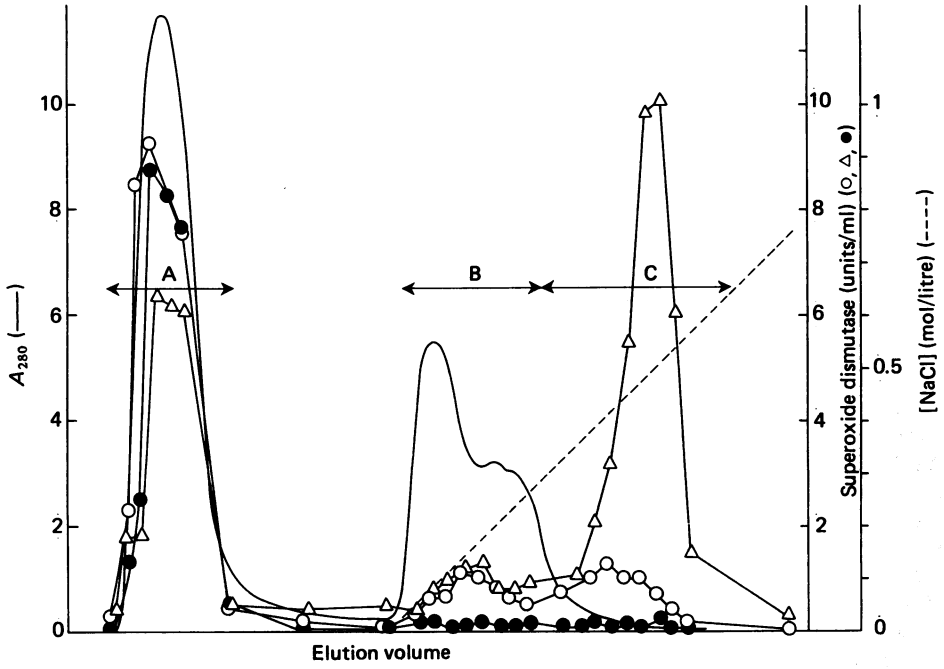

Fig. 3. Separation of plasma on heparin–Sepharose

Human plasma collected before (○) and 10 min after intravenous injection of heparin (200 i.u./kg body weight) (△) was separated on heparin–Sepharose as described in the Materials and methods section. ●, Chromatography of the pre-heparin plasma sample after pretreatment with anti-(EC-SOD)–Sepharose to remove EC-SOD. The continuous line without symbols represents A_{280} and the broken line the NaCl gradient. EC-SOD fractions A, B and C were determined in pools as indicated in the Figure. The activity in pools B and C represents only EC-SOD, since all activity was adsorbed by anti-(EC-SOD)–Sepharose. Pool A contains also Cu,Zn-SOD and cyanide-resistant SOD activity. The EC-SOD activity in fraction A was therefore determined with immobilized antibodies as described for plasma in the Materials and methods section. The EC-SOD activities in fractions A, B and C were: in pre-heparin plasma, 5.2, 4.4 and 5.1 units/ml, and in post-heparin plasma, 7.7, 5.7 and 29.4 units/ml. The recoveries of EC-SOD activity in the chromatograms were 84 and 83% respectively. Note that the larger total SOD activity in pool A in the pre-heparin plasma chromatogram is due to haemolysis in the sample with release of Cu,Zn-SOD from erythrocytes.

activate EC-SOD, nor can any release from blood cells be demonstrated, pointing to the endothelial cells as the most likely source of released EC-SOD. A number of other factors with affinity for heparin, lipoprotein lipase (Krauss *et al.*, 1973; Huttunen *et al.*, 1975), hepatic lipase (Krauss *et al.*, 1973; Huttunen *et al.*, 1975), diamine oxidase (Hansson *et al.*, 1966) and platelet factor 4 (Dawes *et al.*, 1978), have been shown to be rapidly released by intravenous heparin. In most of these cases there is evidence that heparin-induced displacement of the protein from heparan sulphate on endothelial-cell surfaces is the explanation for the phenomenon (Busch *et al.*, 1980; Robinson-White *et al.*, 1985). It appears likely that the release of EC-SOD has the same explanation.

No distinct plateau in the release was reached for heparin doses up to 200 i.u./kg body weight, showing that more heparin is needed for maximal release of EC-SOD than for lipoprotein lipase (Krauss *et al.*, 1973; Huttunen *et al.*, 1975), diamine oxidase (Hansson *et al.*, 1966), hepatic lipase (Krauss *et al.*, 1973; Huttunen *et al.*, 1975) and platelet factor 4 (Randi *et al.*, 1984). The ratio between the affinity for heparin and the binder (heparan sulphate?) on vessel endothelium might be lower for EC-SOD than for the other proteins.

Basal human plasma contains nearly equal amounts of EC-SOD fractions A, B and C. Intravenous heparin released only the high-heparin-affinity fraction, C, which thus appears to be the form which has affinity for endothelial-cell surfaces. The increase achieved here was 4–6-fold, but it is likely that higher doses of heparin would have resulted in a higher ratio. Much higher ratios are achieved for lipoprotein lipase (Krauss *et al.*, 1973; Huttunen *et al.*, 1975), hepatic lipase (Krauss *et al.*, 1973; Huttunen *et al.*, 1975), diamine oxidase (Hansson *et al.*, 1966) and platelet factor 4 (Dawes *et al.*, 1978). Compared with the binding of these proteins, the endothelial binding of EC-SOD appears rather loose. Possibly an equilibrium exists for EC-SOD fraction C between plasma and endothelial-cell surfaces. Most EC-SOD in the vascular system is apparently located on the endothelial-cell surfaces. It should be noted that our data cannot tell from where in the vasculature the EC-SOD is released; it is possible that endothelium in different areas contain different amounts of EC-SOD.

The molecular background for the difference in heparin-affinity between EC-SOD fractions A, B and C is still unresolved. The amino acid and subunit compositions are not significantly different (Marklund, 1982). Nor could any antigenic differences be detected (Marklund, 1984*a*). The binding to the negatively charged heparin is apparently not of a general ion-exchange nature, since no difference between fraction A and C could be detected upon ion-exchange chromatography, and their isoelectric points are identical (pH 4.5; S. L. Marklund, unpublished work). The difference is not due to degradation *in vitro*, since storage of plasma for 3 days in a refrigerator did not change the elution pattern on heparin–Sepharose. Although degradation *in vivo* is a possibility, one might speculate that fractions A and B are specifically intended for protection of fluid components, and C is for shielding cellular surfaces.

Most cell types in the body possess heparin sulphate and other sulphated glucosaminoglycans on their surfaces (Höök *et al.*, 1984). It is possible that much of the EC-SOD found in tissues (Marklund, 1984*b*) is located on such substances on cell membranes and in the connective tissue. The binding of EC-SOD to cellular surfaces might be an especially efficient way of protecting cells against external superoxide radicals. The acid form, $HO_2^{\cdot}$, of the superoxide radical is much more reactive than the base form, $O_2^{-\cdot}$, and can e.g., attack polyunsaturated fatty acids (Bielski, 1983). In the microenvironment of the polyanionic biological membrane surfaces the pH is much lower than in the surrounding solvent (Freeman & Crapo, 1982), favouring the formation of $HO_2^{\cdot}$. Toxic effects of $HO_2^{\cdot}$ have been suggested to be an important mode of superoxide-radical-induced toxicity (Bielski, 1983). Association of SOD with cellular membranes would seem to be a logical way of protecting against such events. It is interesting to note that substitution of Cu,Zn-SOD with polylysine to facilitate association with negatively charged cell membranes highly potentiated the ability of the enzyme to protect activated polymorphonuclear leucocytes against self-inactivation (Salin & McCord, 1977). The cell-membrane-associated SOD of *Nocardia asteroides* confers efficient protection to the bacterium against activated polymorphonuclear leucocytes (Beaman *et al.*, 1985). Micro-organisms lacking affinity for EC-SOD C would, unlike cells in the body with affinity, not benefit from protection by the enzyme. Putative useful functions of superoxide radical, like induction of chemotactic substances in plasma (Petrone *et al.*, 1980), would possibly be less influenced by EC-SOD bound to cell surfaces than by a similarly-cell-protective amount of EC-SOD in solution in plasma.

There is evidence that superoxide radicals produced by activated leucocytes and also by other cell types under certain conditions can directly, or by indirect means, induce chromosomal damage (Emerit, 1983; Birnboim & Kanabus-Kaminska, 1985; Weitberg *et al.*, 1985) and promote carcinogenesis (Borek & Troll, 1983; Nakamura *et al.*, 1985). The surface-associated EC-SOD C would be an efficient protector against such events *in vivo*. In most test systems *in vitro* much of the EC-SOD C would probably be lost from the cells, since the binding appears to be weak. Findings in such systems are then not necessarily quantitatively predictive for the risk of the superoxide-radical-induced damage occurring *in vivo*.

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