Plasma Clearance of Human Extracellular-Superoxide Dismutase C in Rabbits

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Abstract

Extracellular-superoxide dismutase (EC-SOD) is heterogeneous in the vasculature with regard to heparin affinity and can be separated into three fractions: A, without affinity; B, with weak affinity; and C, with relatively strong heparin affinity. The plasma clearance of intravenously injected ^{125}I -labeled and unlabeled human EC-SOD C was studied in rabbits. About 90% of injected ^{125}I -EC-SOD C was eliminated from the blood within 5–10 min. Injection of heparin after 10 or 20 min led to an immediate release of all sequestered ^{125}I -EC-SOD C back to the blood plasma. Later injections of heparin led to diminished release, although release could still be demonstrated after 72 h. A half-time of ~ 10 h could be calculated for heparin-releasable ^{125}I -EC-SOD C. Unlabeled EC-SOD C, determined as enzymic activity and with ELISA, was likewise sequestered and released to the same degree as ^{125}I -labeled EC-SOD C by heparin as tested at 20 min and 5 h. The immediacy of the heparin-induced release indicates that the sequestered enzyme had been bound to endothelial cell surfaces. The length of the half-time suggests that the putative cell surface binding has a physiological function and is not primarily a step in enzyme degradation. The distribution of sequestered ^{125}I -labeled EC-SOD C to different organs was determined at times between 10 min and 24 h. Of the organs, the liver contained the most ^{125}I -EC-SOD C, followed by kidney, spleen, heart, and lung. At all investigated times, the content in the analyzed organs was nearly as large as the amount that could be promptly released to plasma by intravenous heparin. This indicates that almost all ^{125}I -EC-SOD C in the organs was present on endothelial cell surfaces and was not bound by other tissue cell surfaces, or was present within the cells.

Introduction

The secretory enzyme (1) extracellular-superoxide dismutase (EC-SOD [EC 1.15.1.1])¹ is the major SOD isoenzyme in extracellular fluids like plasma, lymph (2), and synovial fluid (3), but also occurs in tissues (4, 5). EC-SOD is a tetrameric, Cu-, and Zn-containing glycoprotein with an apparent subunit molecular weight of 30 kD (1, 6). Plasma EC-SOD from man (7) and other mammals (8) is heterogeneous with regard to affinity

to heparin-Sepharose and can be separated into three fractions: A, without affinity; B, with weak affinity; and C, with relatively strong affinity. Intravenous injection of heparin in man (7) and most mammals (8) leads to a prompt increase in plasma EC-SOD activity. Fraction C is the released form of EC-SOD, and endothelial cell surfaces are the likely source of released enzyme (7, 8). These investigations hinted at the possibility that EC-SOD A and B are circulating forms in the vasculature, whereas fraction C forms an equilibrium between the plasma phase and endothelial cell surfaces. As a further contribution to understanding the behavior of EC-SOD in the vasculature, we here report on the plasma clearance and organ sequestering of ^{125}I -labeled and native human EC-SOD C injected intravenously into rabbits.

Methods

Hypnorm vet. (Fluanison plus Fentanyl) was obtained from AB Leo, Helsingborg, Sweden. Pig gut mucosa heparin (sodium salt) was obtained from KabiVitrum AB, Stockholm, Sweden. The specific activity was 160 IU/mg. Heparin-Sepharose, Sephadex G-50, and Sephacryl S-300 were products of Pharmacia Fine Chemicals, Laboratory Separation Division, Uppsala, Sweden. Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenyl glycoluril) was obtained from Pierce and Warriner Ltd., Chester, England. Na^{125}I in NaOH (100 mCi/ml), $\sim 90\%$ isotopically pure, was obtained from Amersham International, Radiochemical Center, Amersham, England.

Animals. Destination-bred Chinchilla ram rabbits of both sexes, age 5–6 mo and weighing 3–6 kg were used. Each rabbit was used for only one experiment. The animals were given 50 mg/liter NaI in the drinking water to speed up the elimination of ^{125}I .

^{125}I -labeling of human EC-SOD C. EC-SOD C isolated from human umbilical cords (1) was labeled with ^{125}I using the Iodogen method (9). 35 μg EC-SOD C and 0.5 mCi Na^{125}I were mixed in 50 μl 0.3 M Na phosphate, pH 7.5, at room temperature in a test-tube coated with Iodogen. To terminate the iodination, 500 μl 50 mM Na phosphate, pH 7.4, was added after 10 min. After a subsequent 15 min, labeled EC-SOD was separated from Na^{125}I by chromatography on a 12-ml Sephadex G-50 column using 10 mM Na phosphate, pH 7.6, in 150 mM NaCl as eluant. Fractions were collected in tubes containing BSA (final concentrations in collected fractions, 0.4%). The first collected high molecular weight radioactive peak was applied at 6 ml/h to a 2-ml heparin-Sepharose column, equilibrated with 50 mM K phosphate, pH 7.4, containing 0.2% BSA. The column was then washed with the phosphate-BSA buffer. After 10 ml, bound ^{125}I -EC-SOD C was eluted with a gradient in NaCl (0–1.2 M) in the phosphate-BSA buffer. A radioactive peak eluting at ~ 0.5 M NaCl was pooled, divided into portions, and stored at -80°C until used for experiments within 4 wk.

Counting of ^{125}I . The ^{125}I activity of samples was counted in a counter (1260 Multigamma; LKB Instruments, Bromma, Sweden). The volume of the samples was always 1 ml (or 1 g), to keep the counting geometry constant.

Plasma clearance of ^{125}I -EC-SOD C in rabbits. About 10×10^6 cpm ^{125}I -EC-SOD C (~ 1 μg EC-SOD C), dissolved in 50 mM K phosphate, pH 7.4, containing 0.2% BSA, was injected into ear veins. Blood samples were tapped at times indicated in the figures into tubes containing EDTA as anticoagulant. Finally, 2,500 IU/kg body wt heparin was injected, and blood samples were tapped at 1, 5, and 15 min

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1. Abbreviations used in this paper: EC-SOD, extracellular-SOD.

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thereafter. After centrifugation at 2,000 g for 10 min, 1 ml plasma was collected and the ^{125}I radioactivity was counted. The plasma proteins were then precipitated by addition of 300 μl 25% wt/vol TCA. After centrifugation, the ^{125}I of the clear supernatant was counted. After correction for volume, the TCA-soluble (non-EC-SOD) ^{125}I activity was subtracted from the plasma ^{125}I activity.

Screening of ^{125}I -EC-SOD C in rabbits. A large amount of ^{125}I -EC-SOD C (66×10^6 cpm) was injected into two rabbits. Blood was tapped into EDTA tubes after 20 min and the plasma (10 ml containing $\sim 0.7 \times 10^6$ cpm) reinjected into other rabbits.

Sequestering of ^{125}I -EC-SOD C in rabbit organs. 15×10^6 cpm ^{125}I -EC-SOD C was injected intravenously. After indicated times, two rabbits were anesthetized with Hypnorm (AB Leo) and as much blood as possible was tapped. Organs were then rapidly cut out, put on ice, weighed, and the radioactivity was counted in 1 g tissue. Thereafter the organs were homogenized with an Ultra-Turrax (Janke and Kunkel KG, Staufen, FRG) followed by sonication, in 3 vol of 50 mM K phosphate, pH 7.4, containing 0.3 M KBr, 3 mM diethylenetriamine pentaacetic acid, 100 kallikrein inhibition units/ml aprotinin, and 0.5 mM PMSF (the latter three components were used to inhibit proteases). ^{125}I was counted in 1-ml extract. 300 μl 25% TCA per milliliter extract was then added, and the TCA-soluble ^{125}I -activity was counted after centrifugation. The TCA-soluble ^{125}I -activity was then subtracted from the ^{125}I -activity in the extracts. To compensate for ^{125}I -EC-SOD C contributed by the blood in the organs, the hemoglobin content of the organ extracts was determined (10). Using the TCA-precipitable ^{125}I -activity of whole blood at the time the animals were killed and the hemoglobin content of the blood, the ^{125}I -EC-SOD C contributed by blood could be calculated and subtracted from the TCA-precipitable ^{125}I -EC-SOD C activity of the organs.

Plasma volume. The plasma volume of the rabbits was determined with Evans blue (11). Several rabbits in each batch (same age and weight) were tested, and the mean values for the batches were used for rabbits not tested. The plasma volumes varied between 34 and 38 ml/kg body wt.

Analysis of SOD activity. SOD was determined with the direct spectrophotometric method using KO_2 (12) with modifications as described in (13). For specific determination of human EC-SOD C in rabbit plasma, the plasma was incubated overnight with (a) rabbit anti-human EC-SOD-Sepharose 4B or (b) Sepharose 4B (sham incubation) (13). After centrifugation, the SOD activity of the plasma samples was determined. Human EC-SOD was taken as *b* minus *a*.

ELISA for human EC-SOD. Quantitation of human EC-SOD was made by double antibody sandwich ELISA. Microtiter plates (Nunc; Nunc, Roskilde, Denmark) were coated with 100 μl /well of a solution containing 16 $\mu\text{g}/\text{ml}$ of polyclonal rabbit anti-EC-SOD IgG antibodies (14) in 50 mM Na_2CO_3 , pH 9.6. After 2 h incubation, the wells were washed and then blocked overnight with 300 μl blocking buffer (10 mM Na phosphate, pH 7.4, 140 mM NaCl, 0.1% wt/vol Tween 20, and 0.5% BSA). For analysis, 50- μl samples, diluted if necessary with blocking buffer, were added to each well and incubated for 2 h. The wells were then washed with blocking buffer, whereupon 50 μl 3 $\mu\text{g}/\text{ml}$ monoclonal anti-EC-SOD antibody B6, H6 (1) dissolved in blocking buffer was added. After 2 h, the wells were washed with blocking buffer followed by addition of 50 μl peroxidase-conjugated rabbit anti-mouse IgG (DAKOPATTS, Copenhagen, Denmark) dissolved in blocking buffer. After another 2 h, the wells were washed and then developed for 10 min with 100 μl 3.7 mM *O*-phenylenediamine and 0.4 mM H_2O_2 in 100 mM Na citrate, pH 5.0. After addition of 100 μl 0.5 M H_2SO_4 , the absorbance at 492 nm was determined in an ELISA processor II (Hoechst Behring, Marburg, FRG). The assay was standardized with human umbilical cord EC-SOD C. EC-SOD concentrations down to ~ 0.25 ng/ml can be determined.

Results

^{125}I -labeling of EC-SOD C. The ^{125}I -Iodogen procedure resulted in an incorporation of ~ 0.03 – 0.04 mol $^{125}\text{I}/\text{mol}$ EC-

SOD C subunit. Only minor inactivation of enzymic activity occurred; the recoveries were $\sim 90\%$. About 85% of the labeled enzyme bound to Heparin-Sepharose and eluted at the same NaCl concentration (0.5 M) as unlabeled EC-SOD C. The molecular weight, as assessed by chromatography on Sephacryl S-300, was unaffected. Other procedures were also tested: labeling with the chloramine T procedure (15) resulted in loss of enzymic activity and change in gel chromatography pattern. The lactoperoxidase procedure (16) resulted in more loss of enzymic activity (30%).

Plasma clearance of ^{125}I -labeled and unlabeled EC-SOD C and effect of heparin. Fig. 1 shows the time course of plasma ^{125}I -EC-SOD C activity after intravenous injection of labeled enzyme. There is an initial very rapid decline to ~ 10 – 15% of the expected activity followed by a much slower elimination. If heparin is injected within 1 h, there is an almost quantitative release of sequestered ^{125}I -EC-SOD C to plasma. Later heparin injections resulted in less release, although a significant effect was still found at 72 h. A half-time of ~ 10 h could be calculated for the heparin-releasable ^{125}I -EC-SOD C activity. The effect of heparin was very rapid, and maximal release was already seen in the samples tapped 1 min after heparin.

To check the validity of the ^{125}I -EC-SOD C data, 2 mg unlabeled EC-SOD C was injected together with labeled enzyme into rabbits (Fig. 2) and determined as enzymic activity and as protein with ELISA. Like labeled EC-SOD C, unlabeled enzyme was also rapidly sequestered from plasma. The initial decline was, however, larger than for labeled EC-SOD C, down to $\sim 3\%$ at 20 min and 2% at 5 h. The effect of intravenous heparin, however, was almost identical. The large dose of unlabeled EC-SOD C had apparently no effect on the plasma clearance of coinjected labeled enzyme. The results of Fig. 2 for ^{125}I -EC-SOD C are not significantly different from those of Fig. 1.

In Fig. 2, the results are also shown when plasma taken from rabbits 20 min after injection of ^{125}I -EC-SOD C is reinjected into other rabbits. The rapid decline here is less extensive, as is the release induced by intravenous heparin. The difference is not due to an effect of rabbit plasma on ^{125}I -EC-SOD C. If ^{125}I -EC-SOD C, added to rabbit plasma to produce the same activity as in the screened plasma, was injected, the results were not significantly different from those of Fig. 1 (data not shown).

Tissue distribution of sequestered ^{125}I -EC-SOD C. Fig. 3 presents the results of analysis of ^{125}I -EC-SOD C in various organs of rabbits at 10 min to 24 h after intravenous injection of labeled enzyme. TCA-soluble ^{125}I and ^{125}I -EC-SOD C contributed by blood in the organs were subtracted from the data. The magnitude of these corrections is shown in Table I. Most enzyme binds to liver, followed by kidney, spleen, heart, and lung. Other investigated organs (muscle, brain, and adipose tissue) contained less activity and could not be reliably analyzed. The binding per gram wet weight is about equal in liver and spleen; the binding in kidney comes next, followed by that in lung and heart, which bind about equally. The activity disappears with nearly the same rates from liver, kidney, and spleen. In heart and lung, some increase possibly occurs during the 24 h studied but this conclusion is uncertain since the compensations were large and increased with time (Table I). A curve describing the combined contents of the studied organs (cf. data in Fig. 3) is nearly superimposable on a curve describing the heparin-releasable ^{125}I -EC-SOD C activity (cf. data in Fig. 1).

Figure 1. Plasma clearance of ^{125}I -labeled human EC-SOD C and the effect of heparin. The figure shows the time course of plasma ^{125}I -EC-SOD C activity after intravenous injection of ^{125}I -labeled enzyme into 18 rabbits. Injection of ^{125}I -EC-SOD C and handling of blood samples were performed according to Methods. At times indicated by arrows, 2,500 IU/kg body wt heparin was injected into two rabbits (only one rabbit at 24 and 25 h). The injections were made at 10, 20, and 60 min; 2, 5, 12, 24, and 25 h; and 2 and 3 d. The data were calculated on the basis of a plasma volume of ~ 36 ml/kg body wt. The dashed line shows the TCA-soluble ^{125}I activity of samples taken just before injection of heparin.

Figure 2. Plasma clearance of ^{125}I -labeled and unlabeled EC-SOD C and reinjection of ^{125}I -labeled enzyme. 2 mg unlabeled EC-SOD C was injected together with ^{125}I -labeled enzyme ($\square$, $\blacksquare$) into two rabbits. The unlabeled enzyme was determined as enzymic activity ($\circ$, $\bullet$) and as protein ($\diamond$, $\blacklozenge$) with ELISA. (--- Δ ---, --- ∇ ---, --- $\blacktriangledown$ ---) Reinjection into three rabbits of plasma taken from two other rabbits 20 min after injection of ^{125}I -EC-SOD C, c.f. Methods. Arrows indicate injection of 2,500 IU/kg body wt heparin. The heparin was injected at 20 min and 5 h into rabbits given unlabeled EC-SOD C and at 20 min, 2 h, and 24 h into the reinjected rabbits. The ^{125}I -EC-SOD C data were corrected for TCA-soluble ^{125}I activity as described in Methods.

Discussion

EC-SOD is apparently evolutionarily related to the CuZn SODs, because it shows a strong sequence homology with the part of the sequence of the CuZn SODs that defines the active site (17). EC-SOD is, however, a secretory enzyme (1) and should therefore have some special adaptations for a function in the extracellular space. One such special feature of EC-SOD C is apparently the affinity for heparin, which seems to have the correlate in vivo of affinity for heparin analogues like heparan sulfate located on cellular surfaces. A cluster of positively charged amino acids in the carboxy-terminal end of EC-SOD C is probably the receptor for heparin/heparin analogues (17).

Intravenously injected ^{125}I -labeled human EC-SOD C was rapidly sequestered from the blood in rabbits. Early injections of heparin led to an immediate release of all sequestered enzyme to plasma. The releasable activity gradually disappeared with a half-time of ~ 10 h. Intravenously injected human unlabeled EC-SOD C reproduced the essential features of the behavior of ^{125}I -labeled enzyme, although the initial uptake was more extensive (Fig. 2). ^{125}I -EC-SOD C previously screened in rabbits demonstrated less initial uptake (Fig. 2). These findings indicate that a minor part of the labeled enzyme had been altered with respect to tendency to be sequestered, but that $> 90\%$ behaved like unlabeled EC-SOD C.

Intravenous injections of heparin in man (7), rabbits and, most other studied mammals (8) led to a prompt release of endogenous EC-SOD C to plasma. The ^{125}I -EC-SOD C of the present study behaves after sequestering apparently like the endogenous enzyme. Human ^{125}I -EC-SOD C was also injected into a number of rats and guinea pigs, and the results were similar to those found for the rabbits (data not presented). The

Figure 3. Tissue distribution of sequestered ^{125}I -EC-SOD C. Results of analysis of ^{125}I -EC-SOD C in various organs of rabbits at 10 min to 24 h after intravenous injection of labeled enzyme. Handling of the tissue samples was done as described in Methods. ---○---, combined ^{125}I -EC-SOD C content of studied organs.

rapid sequestering and immediate release to plasma brought about by heparin indicates that the bound enzyme must be located very close to the blood stream. The most likely site is endothelial cell surfaces. Given the large pores in the sinusoids, hepatocytes are also possible sites in the liver. For several other plasma factors with heparin affinity (like lipoprotein lipase, hepatic lipase, and platelet factor 4) that are rapidly released by intravenous heparin, there are indications that the endothelial cell surface receptor is the heparin analogue heparan sulfate (18, 19). It is probable that heparan sulfate is also the receptor for EC-SOD C. Heparan sulfate is found on the surface of most cell types in the body (20). The effect of the intravenous heparin is then due to binding to the receptor for heparin/heparan

sulfate on EC-SOD C, resulting in release from endothelial cell surfaces.

As for the distribution of sequestered ^{125}I -EC-SOD C, most enzyme bound to the liver, followed by, of the investigated organs, kidney, spleen, heart, and lung. The sum of the sequestered activity in these organs (Fig. 3) was nearly as large as the heparin-releasable ^{125}I -EC-SOD C activity at all investigated times (Fig. 1). Consequently, at least during the first hour, when almost all sequestered activity could be returned to plasma by heparin, the ^{125}I -EC-SOD C in the studied organs must be bound close to the blood, i.e., on vessel endothelium. Furthermore, no major binding to other tissues can exist. Because we lack information on the overall balance of ^{125}I in the rabbits, these conclusions cannot be drawn with certainty at times beyond 1 h but are also probably valid there.

Apparently, the injected human EC-SOD C forms an equilibrium between the plasma phase and endothelial cell surfaces. The ratio between dissolved EC-SOD C and surface-bound enzyme in the vasculature appears to be $\sim 1:30$ (data for unlabeled enzyme, Fig. 2). This figure is larger than the ratio ($\sim 1:10$) that can be calculated from the release of endogenous EC-SOD C in man after a large dose of intravenous heparin (7).

The occurrence of TCA-soluble ^{125}I activity in plasma (Fig. 1) shows that the EC-SOD C is gradually degraded. The major site(s) of EC-SOD C degradation cannot be deduced from the present data. The liver would seem to be a likely candidate, but since bound EC-SOD C is apparently in equilibrium with the dissolved enzyme, redistribution to other potential degradatory sites is possible. The putative surface association of EC-SOD C in the circulation is probably not primarily a step in the degradation of the enzyme, since the half-time of bound enzyme is so long (Fig. 1). For comparison, lipoprotein lipase is also rapidly bound to the liver after intravenous injection. The enzyme can partly be released by heparin, but the half-time of releasable enzyme is only 10 min (21). It is probable that the cell surface association of EC-SOD C has an important physiological function. Note that CuZn SOD that is substituted with polylysine to facilitate association with negative charges on cell surfaces is much more efficient than native CuZn SOD in protection of activated neutrophil leukocytes against O_2^- -induced autoinactivation (22). The cell surface-associated SOD in *Nocardia asteroides* has been shown to be important for the protection of the bacterium against activated neutrophil leukocytes (23).

Table I. Compensation for TCA-soluble ^{125}I and ^{125}I -EC-SOD C in Blood in Investigated Organs

	10 min		20 min		60 min		2 h		12 h		24 h		Organ weight g
	A	B	A	B	A	B	A	B	A	B	A	B	
Liver	0.4	6.9	0.2	6.9	0.4	9.3	0.3	7.2	1.2	9.0	0.6	13.4	155 $\pm$ 36
Kidneys	1.3	11.9	1.2	11.6	1.4	27.2	0.4	31.8	1.4	41.9	0.4	50.8	29.4 $\pm$ 2.9
Spleen	1.6	8.2	1.8	7.4	2.0	7.8	3.7	6.6	11.4	12.1	8.5	15.2	2.2 $\pm$ 0.5
Heart	18.0	31.5	12.6	31.8	5.4	57.2	7.2	50.2	2.4	62.2	2.8	68.4	11.2 $\pm$ 1.7
Lungs	51.5	25.4	51.1	30.7	45.2	42.3	10.4	51	12.4	64.8	9.2	72.8	17.8 $\pm$ 3.9

The ^{125}I -EC-SOD C activity in rabbit organs (cf. Fig. 3) was compensated for (A) organ ^{125}I activity soluble in TCA and (B) ^{125}I -EC-SOD C content in the blood of the organs as described in Methods. The table shows the magnitude of the compensations expressed as percent of the total ^{125}I -activity in the organs at different times after injection. The mean weights ($\pm$ SD) of investigated organs are also presented.

The major tissue location of sequestered human EC-SOD C in rabbits was the liver. This organ in rabbits is apparently equipped with abundant high affinity binding sites for EC-SOD. Unlike CuZn SOD and Mn SOD, the tissue contents of EC-SOD show a high variability between mammalian species (5). It is therefore possible that injected EC-SOD C would show a different sequestering pattern in other species.

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