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HEPARIN, A POTENT RELEASING AGENT OF EXTRACELLULAR SUPEROXIDE DISMUTASE (EC-SOD C), SUPPRESSES ISCHAEMIC PAW OEDEMA IN MICE

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Heparin (2,000 U/kg, i.v.) increases the plasma superoxide dismutase (SOD) activity by 2–3 times after 5 min. followed by a gradual decrease. A high dose of heparin (4×10^3 and 10×10^3 U/kg) exhibits a lower increase in the release of SOD. Ischaemic paw oedema in mice was suppressed by various types of SOD and heparin also suppresses this oedema. The dose-dependent curve of heparin of oedema suppression corresponds well with the increased plasma level of SOD. Inducibility with heparin, slow clearance from the bloodstream and blocking of oedema suppression by the copper chelator, diethyldithiocarbamate (DDC), suggest that the oedema suppressing SOD was the extracellular (EC)-SOD C. Other anticoagulants such as citrate and EDTA had no effect. Chondroitin sulphate A and C or carrageenan exhibited weak suppression. A complex of EC-SOD C and heparin appears not to bind to the endothelium in contrast to the injected free EC-SOD C. When heparin is re-injected, more than 1 week was required to get the same degree of oedema suppression. This indicates the necessity of newly synthesized enzyme. A biological role for heparin-induced release of plasma SOD is demonstrated for the first time in this investigation.

KEY WORDS: Heparin, extracellular superoxide dismutase, ischaemia, mice.

Heparin is a natural anticoagulant. However, many biological effects ascribed to it can only be interpreted by other functions of this sulphated polysaccharide. Serum lipid clearing factor was shown to be a lipoprotein lipase that can be split off from the endothelium by heparin.[1,2] Many sulphated polysaccharides without anticoagulant activity also release this lipase into the plasma.[3] Another example is diamine oxidase (DAO) release in rats by heparin (i.v., 4,000 U/kg).[4] Rabbits release less DAO than guinea pigs when injected with 200 U/kg heparin.[5]

Cytosolic Cu,Zn-SOD and mitochondrial Mn-SOD can be detected in the plasma but they are believed not to protect blood vessels from any oxidative stress. They arise from various organs and pass through the circulatory system only as turnover products. Ascorbate, α-tocopherol, urate, bilirubin as well as caeruloplasmin have been reported as putative protective agents for plasma and blood vessels.[6] However, Marklund[7] has demonstrated the presence of a high molecular mass extracellular superoxide dismutase (EC-SOD), containing copper. Further investigations demonstrated that 3 types of EC-SODs are present.[8] EC-SOD A has no affinity to heparin or the endothelium, and EC-SOD B shows weak binding affinity to both. These two SODs can protect only plasma, but EC-SOD C is believed to protect also the blood vessels where abundant amount of superoxide radicals (O_2^-) can be produced by attached neutrophils or by activated xanthine oxidase (XOD). EC-SOD C has strong

affinity to the heparinoids and may exist bound to the sulphated proteoglycans on cell surfaces or in the intracellular matrix.[9] Intravenous (i.v.) injection of heparin (2,000 U/kg) rapidly removes the bound EC-SOD C from the cell and covers the surfaces of blood vessels to protect them from any oxidant attack.[8] Positively charged C-terminal amino acids of EC-SOD C bind strongly to the negatively charged heparin. Distribution of injected human EC-SOD C was also tested in rabbit organs.[9] However, any biological effect of EC-SOD C has not yet been investigated.

In our previous work, the intravenously injected SODs from various animals suppressed rather specifically the ischaemic paw oedema of mice.[10] Consequently if heparin increases the plasma SOD level, then the paw oedema might also be suppressed. We examined this possibility with different doses and different times of injections in mice. Suppression of ischaemic paw oedema corresponds well with the measured increase of plasma SOD activity. A possible role and movement of EC-SOD C *in vivo* is discussed.

Heparin (25,000 U/kg, i.v.) suppresses the rabbit Arthus reaction[11] and 3,600 U/kg heparin protects against the anaphylactic shock of rabbit which accompanies serotonin release.[12] Many clinical applications including for angina pectoris have also been tried.[13] Autoimmune diseases or allograft rejection is reported to be suppressed by heparin.[14] Local administration of heparin polymer is claimed as a useful surgical tool.[15] Heparin fragments or non-anticoagulant sulphated polysaccharides such as β-cyclodextrin tetradecasulphate suppress tumor angiogenesis when given with steroids.[16,17] Some of these functions of heparin and heparinoids might also be related to their binding capacity with EC-SOD C on the endothelium.

MATERIALS AND METHODS

Blood sampling

Haemolysis was carefully avoided for blood sampling because erythrocytes contains 100 times more SOD than serum and a little release of SOD from erythrocytes influences the plasma SOD content. Cardiac puncture is not only time consuming but also results in increased possibility of haemolysis, therefore blood was obtained by cutting the aorta at the throat. Mice of the same strain were used for testing ischaemic paw oedema. About 0.5 ml of blood was collected in plastic tubes containing 2.0 ml of 0.25 M sucrose. This medium also contained 10 U/ml heparin which showed no effect on SOD activity *in vitro*. When 2,000 U/kg heparin were injected, it was calculated that only a maximum of 8 U/ml resulted in the assay sample. A ten fold increase in the injected sample resulted in 80 U/ml in the assay sample. These concentrations of heparin do not have any effect on SOD assay, because final concentrations of 10, 20, 100 and 200 U/ml had no influence. After repeated trials, 0.25 M sucrose was chosen as the best medium for minimum haemolysis when kept on ice (0–4°C). Sodium chloride solution (5 and 10%) resulted in some haemolysis while bovine serum albumin (BSA, 1 and 5%) and adenosine triphosphate (ATP)-2Na (10^{-4} and 10^{-3} M) resulted in strong haemolysis. Even when using 0.25 M sucrose, haemolysis sometimes occurred as a result of an unknown factor. Haemolysed samples were discarded and only clear samples were utilized for SOD determination.

As it was difficult to get exactly 0.5 ml of blood each time, the obtained blood volume was collected by measuring the haemoglobin concentration in the blood

before centrifugation. Twenty μl of blood were mixed with 2.0 ml of 0.25 M sucrose and diluted with 10.0 ml saline. The haemoglobin concentration was determined by the absorption at 418 nm. Standard blood sampling was run only for this purpose. When exactly 0.5 ml were taken from normal mice, this resulted in an absorption of 0.506 $\pm$ 0.005 from 5 determinations. Each plasma sample was corrected by multiplying $\sqrt{f}$ to the obtained IC_{50} where $\sqrt{f}$ is ($O.D._{418\,nm}$ of test sample)/0.506. IC_{50} can be obtained from the graph of O_2^- suppression (vertical normal scale) versus plasma concentration (horizontal log scale), so $\sqrt{f}$ times of plasma sample were required to get IC_{50} when exactly 0.5 ml blood was taken.

Assay of SOD activity

Plasma obtained by centrifugation (3,000 rpm., 15 min., 0–4°C) was carefully transferred by pipetting into the reaction tubes. Two samples each of 300, 200, 100, 50 and 25 μl plasma were prepared for determination by the nitrite method.[18] Volumes were adjusted to 300 μl by the addition of 0.25 M sucrose with 10 U/ml heparin. 100 μl of 10 mM hydroxylamine hydrochloride, 100 μl of 4 mM hypoxanthine, 200 μl of buffer solution (20 mM borate and 30 mM KH_2PO_4, pH 8.2, containing 1 mM EDTA) were added as well as 100 μl water to a final volume of 800 μl.

To one series of diluted plasma tubes were added 200 μl xanthine oxidase (XOD) (5–40 mU) and incubated at 37°C for 30 min. with gentle shaking. The XOD concentration must be controlled for each experiment, because the O_2^- producing capacity was found to differ from batch to batch. Control difference of absorption at 550 nm (O.D. from added XOD − O.D. from minus XOD) was kept to between 0.250 and 0.300 by changing the concentration of XOD before each assay. SOD activity was apt to increase when tested under weak O_2^- producing condition.

The reaction was stopped by the addition of 2.0 ml of 25% (w/v) acetic acid which contained 30 μM N-1-naphthyl-ethylenediamine dihydrochloride and 450 μM sulphanilic acid. The tubes were allowed to stand for 30 min. to 3 hrs. at room temperature before the absorption at 550 nm was measured. Another series of reaction mixtures served as blanks. Two hundred μl of water were added instead of XOD. Each $O.D._{550\,nm}$ (plus XOD − minus XOD value) was divided by that of the control for measurement of percentage inhibition (Table 1).

Effect of plasma protein on SOD activity

Plasma proteins are known to have some O_2^- scavenging effect.[18] By testing the inhibition by a model protein such as BSA, plasma proteins were supposed to contribute 15% to the observed determination from SOD containing plasma samples. Net SOD inhibition at each diluted plasma, IC_{50} by SOD must be always right-shifted on the log concentration scale by a degree of (100%/100%−15% = 1.346).[2] One nitrite unit (NU) is the plasma volume (ml) which is able to scavenge 50% of O_2^-, so that the SOD activity (NU/ml) can be calculated backwards (Table 1). The nitrite unit differs from the cytochrome c unit (CU) of McCord and Fridovich[19] and the potassium superoxide (KO_2) disproportionation method of Marklund.[20]

The nitrite method is about 9 times more sensitive to both the Cu,Zn-SOD and Mn-SOD.[18] Sigma bovine Cu,Zn-SOD (3,000 U/mg) gave an IC_{50} of 0.04 μg/ml in a 1.0 ml reaction mixture by the nitrite method. This results in 25,000 NU/mg which is 8.3 times higher than CU. Interference by other proteins was 10 fold less in this

TABLE I

Example of SOD activity assay of plasma by the nitrite method. Plasma from control(A) or heparin-injected(B) mice was diluted as indicated. Inhibitions by these dilutions were obtained with plus XOD and minus XOD tubes. Apparent IC_{50}(e) as plasma volume per ml of reaction mixture was read from the graph followed by the correction of plasma protein(g) and blood sampling volume(h). SOD activity as nitrite units(NU)/ml plasma(i) was calculated by inverting the net IC_{50} value.

Sample of 20 min after injection (i.v.) of	Final plasma conc.	Measured O.D. 550 nm			Inhibition	Direct reading	Protein effect corrected	Sample volume corrected	SOD activity
		+XOD	−XOD	a-b	%	(graph)	1.346·e	$\sqrt{f}$·g	100/h
		a	b	c	d	e	= g	= h	= i
	0	0.310	0.044	0.266			$\sqrt{f(A)}$ = 0.812		
	0	0.327	0.048	0.279	(0%)		$\sqrt{f(B)}$ = 1.269		
				AV. = 0.273					
	6% (0.3 ml)	0.162	0.039	0.123	55				
	4% (0.2 ml)	0.180	0.041	0.139	49				
(A)	2% (0.1 ml)	0.220	0.046	0.174	36	IC_{50}(A)	IC'_{50}(A)	IC''_{50}(A)	22.3 NU/ml
Saline	1% (0.05 ml)	0.229	0.041	0.188	31	= 4.10	= 5.52	= 4.48	plasma
(B)	4% (0.2 ml)	0.143	0.032	0.111	59				
Heparin	2% (0.1 ml)	0.160	0.036	0.124	55	IC_{50}(B)	IC'_{50}(B)	IC''_{50}(B)	64.9
2,000	1% (0.05 ml)	0.165	0.032	0.133	51	= 0.90	= 1.21	= 1.54	NU/ml
U/kg	0.5% (0.025 ml)	0.192	0.039	0.153	44				plasma

method. One SOD unit determined by the KO_2 method corresponded to 8.3 ng Cu,Zn-SOD and 65 ng Mn-SOD. This is about 40 and 5 times more sensitive respectively than the cytochrome c method.[8]

Assay for ischaemic paw oedema

Saline or drug solution (0.5 ml) was injected intravenously (by the tail) to mice (male ddY strain, 27–33 g, Shizuoka Agr. Corp. Assoc.) and the right hind leg was bound 12 times with a commercial rubber ring (1 × 1 mm, d = 42 mm) at just above the articulation.[10] A mouse was placed in a plastic cylinder device being picked up the right hind leg from the slit (Figure 1). The bound rubber ring was scissored off after 20 min. using the same device, and then immediately the paw thickness was measured with a Citizen Thickness gauge (Citizen Watch Co., Tokyo). The swollen paw as measured 20 min. after the recirculation. Each test group consisted of at least 4 mice and the mean value of increased thickness was divided by that of control to get the percentage of suppression. The mean ± S.E. (standard error) was obtained following triplicate experiments. Paw thickness increased time-dependently until 30 min. Gain of thickness over normal paw thickness was 0.7 to 0.8 mm after 20 min. ischaemia and 20 min. recirculation.

Chemicals

Heparin sodium (178.9 U/mg, porcine intestinal mucosa), chondroitin sulphate A sodium (bovine trachea) and chondroitin sulphate C sodium (shark cartilage) were obtained from Nakarai Chem. Co. (Kyoto). Carrageenan (type IV), Cu,Zn-SOD (bovine erythrocyte, 3,000 U/mg) and bovine serum albumin (BSA) were the products of Sigma Co. Milk xanthine oxidase (XOD, 20 U/ml suspension) was purchased from Boehringer Mannheim GmbH (West Germany). All other chemicals were analytical grade.

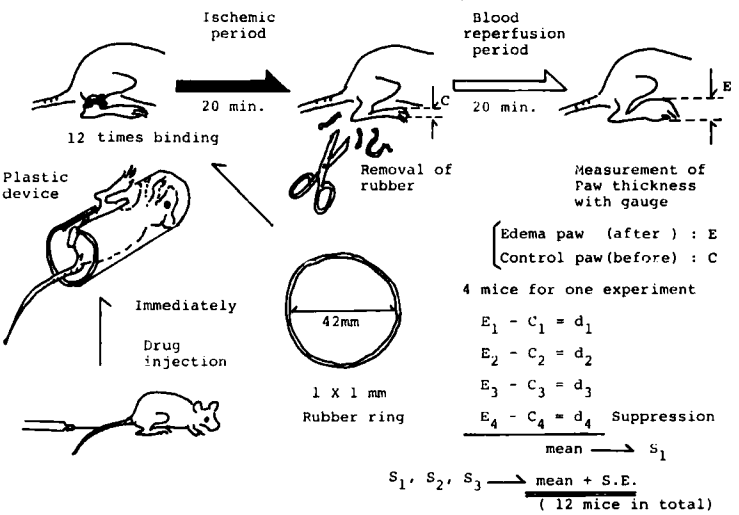


FIGURE 1 Procedure for ischaemic paw oedema assay.

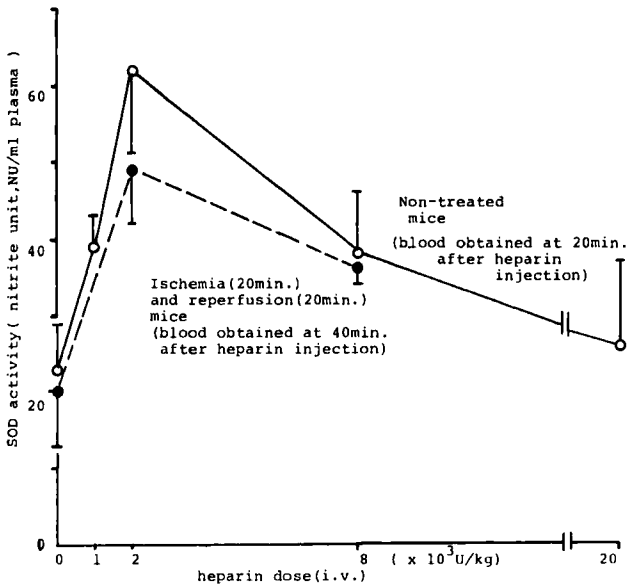


FIGURE 2 Increase of plasma SOD activity by different doses of heparin. White circle is for non-treated mice. Blood was obtained at 20 min. after heparin injection. Black circle is for ischaemia-recirculation mice. Blood was obtained at 40 min. after heparin injection. Mean ± S.E. of 3 experiments with 4 mice (12 mice in total).

RESULTS

Increase in plasma SOD activity caused by heparin

As the paw oedema had started to develop at 20 min (the beginning of recirculation time) after the drug injection, the increase of plasma SOD activity at this time was examined. Figure 2 shows that 2,000 U/kg heparin caused the most potent increase of plasma SOD both in non-treated and ischaemia-recirculation mice. A slightly lower activity in ischaemia-recirculation mice (Figure 2) was due to the retarded time of blood sampling (40 min.). Normal mice showed essentially the same curve at 40 min. (Figure 3), so that ischaemia-recirculation of hind paw resulted in no influence on the increase of plasma SOD activity by heparin. The very rapid increase in plasma SOD activity caused by heparin is shown in Figure 3. At 5 min. the SOD level increased to 74 NU/ml from 21 NU/ml (3.5 fold increase) followed by a gradual decrease. The SOD activity at 120 min. was even lower than that of normal mice. An increase in the plasma SOD activity with heparin (2.2 times) was also reported by Karlsson and Marklund.[8]

Effect of heparin on ischaemic paw oedema

Ischaemic paw oedema of mice was most suppressed by 2,000 U/kg heparin (Figure 4). The dose-response curve of ischaemia-recirculation paw oedema corresponded very well with the increase in plasma SOD activity. When the Cu,Zn-SOD inhibitor, sodium diethyldithiocarbamate (0.2 g/kg, i.v.) was co-injected, the increase of plasma

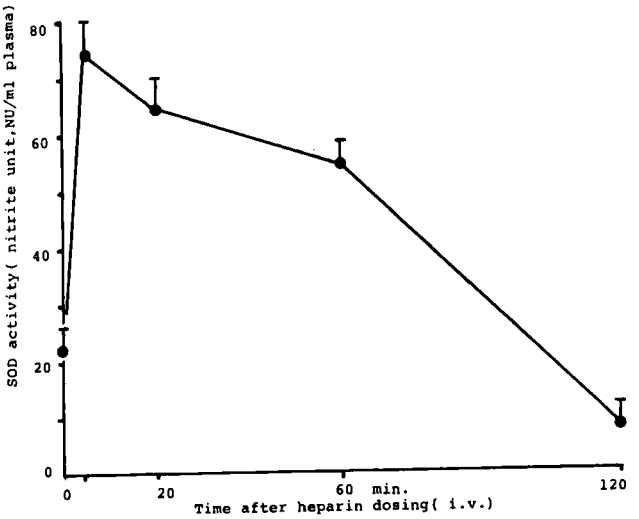


FIGURE 3 Time course of plasma SOD activity of non-treated mice after 2,000 U/kg heparin injection. Mean ±S.E. of 3 experiments with 4 mice (12 mice in total).

SOD activity was blocked, so that the plasma SOD was presumed to be a copper-containing SOD. Diethyldithiocarbamate is also an SH agent and an immuno-modulator, but the lack of effect of reduced glutathione (data not shown) and its rapid effect excludes the possibility that diethyldithiocarbamate functions in this manner. Injected cytosolic bovine Cu,Zn-SOD has been reported to have a short half life ($t_{1/2}$ = 6 min.) in the plasma because of its low molecular mass (32,000 dalton).

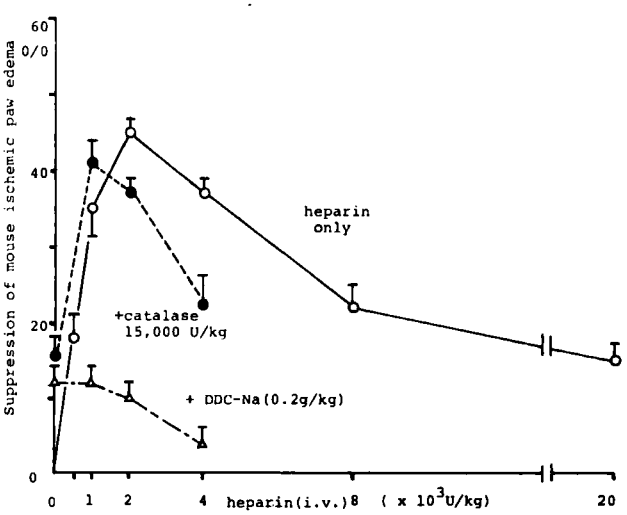


FIGURE 4 Suppression of ischaemic paw oedema of mouse by different doses of heparin. Heparin alone or mixed with drug (0.5 ml/30 g body weight) was injected just before ischaemia. Mean ±S.E. of 3 experiments with 4 mice (12 mice in total).

This fact was also confirmed in our previous report on the suppressive effect by this form of SOD on ischaemic paw oedema of mice.[10] A slower decrease of SOD activity after 5 min (Figure 3) suggests that the heparin-induced plasma SOD activity is not the cytosolic Cu,Zn-SOD, but copper-containing heparin bound EC-SOD C of high molecular mass (135,000 dalton) that could not be easily excreted. Injection (i.v.) of catalase neither suppressed the oedema nor enhanced the suppressive effect of heparin. The reason for the small shift in the peak of oedema is not clear.

Effect of heparin-like compounds

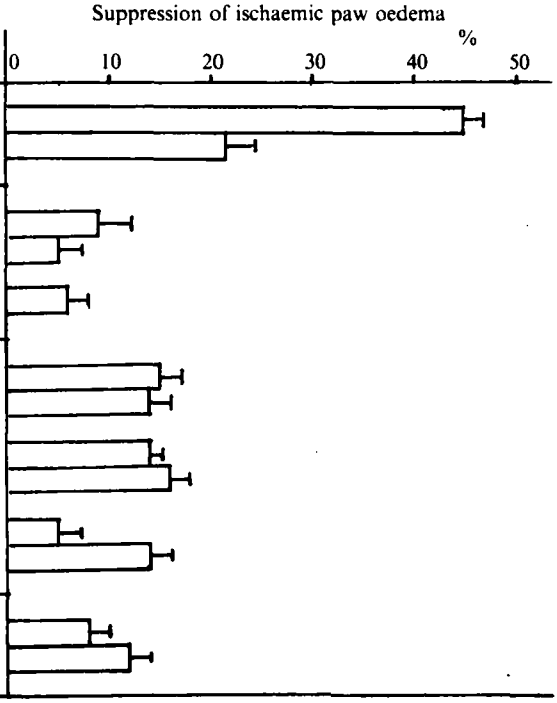
It is possible that the anticoagulant activity of heparin may have no relationship to the observed suppression of the ischaemic oedema because high doses of other anticoagulants such as sodium citrate or EDTA had no effect (Table 2). However, sulphated polysaccharide sulphates A and C or carrageenan only suppressed this ischaemic oedema to a smaller extent than heparin. The hydroxyl radical (·OH) scavenger, dimethylthiourea (DMTU) failed to suppress the oedema indicating that ·OH was not involved in the model utilized.

Since injection of heparin resulted in a very rapid increase of EC-SOD C in plasma, this situation could be interpreted as similar to when isolated mouse EC-SOD C is

TABLE 2

Suppression of ischaemic paw oedema. Drug was injected just before ischaemia. Mean ± S.E. of 3 experiments with 4 mice (12 mice in total).

Agent (i.v.)	Dose	Suppression of ischaemic paw oedema (%)
Heparin	× 1 (11.2 mg/kg)	
	× 4 (44.7 mg/kg)	
Citrate-Na	× 1 (11.2 mg/kg)	
	× 10 (112 mg/kg)	
EDTA-Na	× 2 (22.35 mg/kg)	
Chondroitin sulfate A	× 1 (11.2 mg/kg)	
	× 10 (112 mg/kg)	
Chondroitin sulfate C	× 1 (11.2 mg/kg)	
	× 10 (112 mg/kg)	
Carrageenan	× 1 (11.2 mg/kg)	
	× 4 (44.7 mg/kg)	
DMTU	× 4 (44.7 mg/kg)	
	× 20 (224 mg/kg)	



Agent was injected just before ischaemia (20 min.) and paw thickness was measured at 20 min. after reperfusion. (n = 3 of 4 mice).

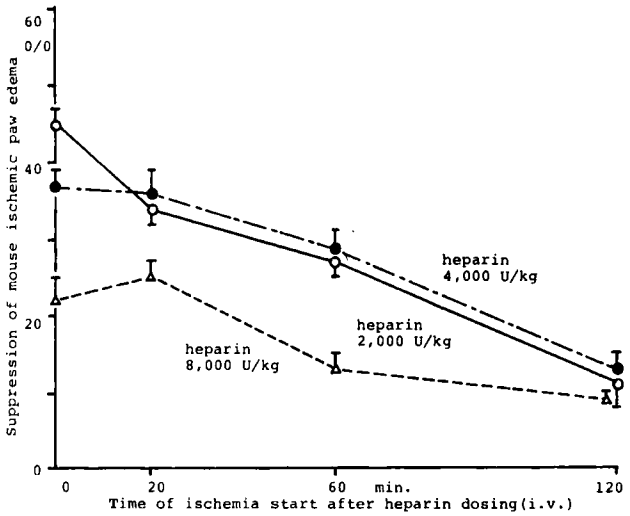


FIGURE 5 Time course of heparin suppressive effect on ischaemic paw oedema. Ischaemia was started at the indicated time after different doses of heparin. Mean ± S.E. of 3 experiments with 4 mice (12 mice in total).

injected. Therefore, the half life of EC-SOD C in plasma could be estimated as being about 70 min. from Figure 3 and Figure 5.

De novo synthesis of EC-SOD C

EC-SOD A and B are reported not to be bound to tissue, and their level in plasma does not increase following heparin injection.[8] Heparin is therefore releasing the EC-SOD C bound to the endothelium. Heparin was injected twice with various time intervals in order to determine the difference in paw oedema suppressing capacity. When 2,000 U/kg heparin was injected, the endothelial EC-SOD C was removed and very little SOD was released by the second heparin injection (Figure 6). EC-SOD C once bound to heparin could not reattach to the endothelium. Full release of EC-SOD C by heparin into the plasma, was achieved after 8 days, suggesting a requirement for *de novo* synthesis. When the first injection dose of heparin was 8,000 U/kg, the recovery to get full release of EC-SOD C, was attained after more than 12 days.

Effect of heparin with SOD

The possibility that heparin activates the endogenous SOD was considered. Injections of bovine Cu,Zn-SOD (32,000 dalton) in various doses were mixed with a moderate dose of heparin. A dose of 500 U/kg heparin by itself suppressed the ischaemic paw oedema by 21%. When 3 and 30 U/kg SOD were injected with heparin, the suppression was 35 and 50%, respectively. These doses of SOD alone suppressed by 17 and 20% indicating that the observed effect from the combined dose is additive. Suppression of the ischaemic paw oedema was again suggested to be due to the increased plasma EC-SOD C rather than the activation of pre-existing lower molecular mass SOD.

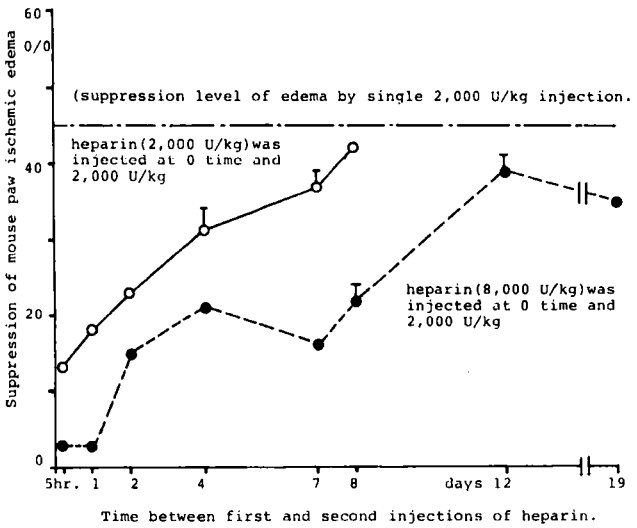


FIGURE 6 Suppression of ischaemic paw oedema by a second injection of heparin (2,000 U/kg). White circles represents mice which received a first injection of heparin (2,000 U/kg) without ischaemic treatment and received ischaemia-recirculation treatment at the indicated time after the first injection. Black circles represents mice which received a first injection of 8,000 U/kg heparin followed by the same treatment. A circle represents mean of 4 mice and bar is S.E. of mean (3 experiments with 4 mice each).

In addition, subcutaneously injected heparin (2,000, 8,000 and 20,000 U/kg) at 2 hr. before onset of ischaemia suppressed the oedema only by 10, 13 and 4% (n = 4). Direct contact of heparin with the endothelial cells seems necessary for increasing plasma SOD level and for suppressing the ischaemic paw oedema.

DISCUSSION

Karlsson and Marklund[8] observed a rapid increase of plasma SOD activity following intravenous injection of heparin (2,000 U/kg) in mammals except rats. Heparin caused about 2.2 times and 5.2 times increase of plasma SOD activity in mice and man, respectively. However, the effect was only observed at this single dose and no evidence was presented that high doses of heparin result in a lower increase in the plasma SOD activity. As we already demonstrated that the ischaemic paw oedema can be suppressed rather specifically by various types of SODs (i.v.),[10] this oedema was expected to be suppressed by heparin-induced plasma SOD. Heparin bound to EC-SOD C ten times more strongly than to the heparan sulphates of endothelium, where this form of the enzyme is originally attached. Therefore, heparin could release EC-SOD C as an enzyme-heparin complex into the plasma. Rats appear to be an exception as heparin does not augment plasma SOD. It was therefore concluded that there is only EC-SOD A and B in rat plasma and endothelium.[8]

Injected human EC-SOD C was reported to be trapped in rabbit organs. Trapped EC-SOD C could be rapidly released again into the plasma by heparin up to 1 day after the enzyme injection.[21] Normal animals may have both vacant carrier and EC-SOD C occupied heparan sulphate carrier, because injected EC-SOD C can be

fixed to the endothelium. It is also possible that this carrier is occupied by other proteins which bind more weakly and can be replaced by EC-SOD C. Judging from the data in Figure 5, when EC-SOD C is released by heparin (2,000 U/kg), the mice could not respond efficiently to a second injection of heparin to release and to suppress the ischaemic oedema until 8 days after. The eight days required for recovery may represent the time for carrier-bound EC-SOD C to be completely synthesized *de novo*. It is not clear whether only EC-SOD C must be resynthesized or both enzyme and carrier must be renewed. Recovery was prolonged when the dose of heparin for the first injection was augmented.

Heparin bound to EC-SOD C cannot apparently attach again as free enzyme to the endothelium with the result that the heparin-EC-SOD C complex itself must protect the endothelium when released before it diffuses in the plasma. A small amount of EC-SOD C and heparin (or natural heparin-like substance) complex might be continuously released from endothelium. As another possibility, the second carrier or receptor for EC-SOD C-heparin complex must be presumed to be on the endothelial surface. Moderately heparin-loaded enzyme may fix to this receptor as an enzymatically active form.

Fig. 7 is an interpretation of the data obtained assuming that one kind of EC-SOD C carrier is working. Injected free EC-SOD C is stable following its binding with vacant heparan sulphate carrier on the endothelium. When an appropriate amount of heparin is injected, its strong affinity to the positively charged part of EC-SOD C (20 amino acids chain of C-terminal including 3 lysine and 6 arginine),[9] functions to detach this enzyme. Heparin bound subunits are possibly the conformationally

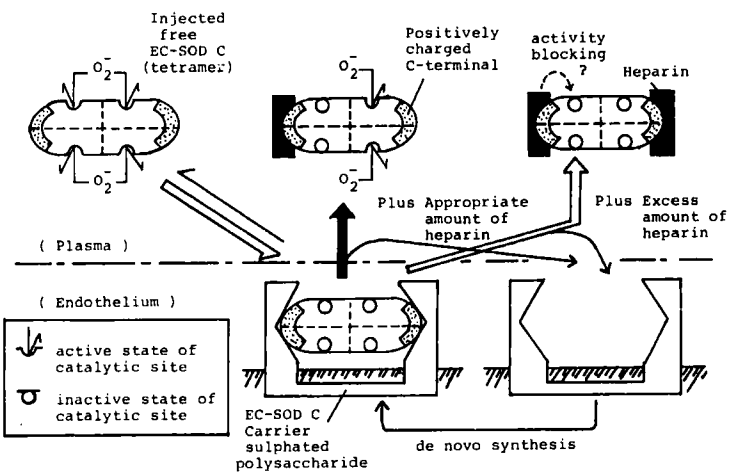


FIGURE 7 A hypothetical mechanism for EC-SOD C release from endothelium to plasma. Endogenous EC-SOD C is fixed with weak affinity to endothelial carrier. Negatively-charged heparin binds with the positively charged C-terminal of this EC-SOD C and splits off into plasma. Heparin binding may block the catalytic activity because excess heparin results in decreased plasma SOD activity. This blocking may be due to the conformational change as a consequence of heparin binding to C-terminal or by direct attachment to the catalytic amino acids. Heparin-induced EC-SOD C cannot rebind to the endothelium, for the binding between the enzyme and heparin is too strong. Carrier free EC-SOD C will be filled with newly synthesized enzyme after more than 1 week. Injected isolated EC-SOD C can attach to the carrier and easily return to the plasma following reaction with heparin.

blocked and cannot express their catalytic activity in the plasma. Consequently if an excess amount of heparin is present in plasma, all 4 subunits of EC-SOD C can bind with heparin resulting in complete blocking of activity. Modification of only one lysine or histidine is reported to cause a decrease in the SOD activity.[22,23]

The main site of oxidant attack is believed to be the endothelium where the adherent neutrophils or activated XOD are producing $O_2^{\cdot-}$. EC-SOD A and B are soluble in the plasma and contribute very little to the prevention of the endothelium. The data obtained could be interpreted by the hypothesis presented in Figure 7. Immunological or other direct experiments may prove whether the one or two carrier theory is the most suitable interpretation.

Further experiments with other models are required to elucidate the EC-SOD C releasing activity of heparin and to study the beneficial clinical application of heparin-like substances.

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