Free Rad. Res. Comms., Vol. 4, No. 6, pp. 385–396
Reprints available directly from the publisher
Photocopying permitted by license only

SUPPRESSIONS OF ISCHEMIC PAW OEDEMA IN MICE, RATS AND GUINEA PIGS BY SUPEROXIDE DISMUTASES FROM DIFFERENT SOURCES

Y. ŌYANAGUI, S. SATO and T. OKAJIMA

Research Laboratories of Fujisawa Pharmaceutical Co. 1–6, 2-Chome, Kashima, Yodogawa-Ku, Osaka 532, Japan

(Received November 6, 1987)

Various sources of superoxide dismutases (SOD) suppressed ischaemic paw oedemata (tourniquet poditis) of mice, rats and guinea pigs with different potencies. Intravenous (i.v.) dosing of mouse Cu,Zn-SOD had no effect on mouse ischaemic oedema, yet rat and guinea pig Cu,Zn-SOD suppressed ischaemic oedemata of rats and guinea pigs. Homologous SOD was anti-inflammatory at least in these two models. Guinea pig SOD was one of the most potent in all models, but showed a very narrow range of effective dose. This bell-shape suppressive pattern was ameliorated by concomitant catalase injection. Bovine and human Cu,Zn-SOD had a rather broad range of effective dose. Bacterial Mn-SODs were suppressive in mice, as well as the oxygen radical scavenger MK-447 and cytochrome c. Dexamethasone was effective only when administered more than 3 hrs in advance. As ischaemic paw oedema of mice was not sensitive to cyclooxygenase and lipoxygenase inhibitors, this model could serve for screening new types of anti-inflammatory or anti-ischaemic drugs.

KEY WORDS: Superoxide dismutase, inflammation, dexamethasone.

INTRODUCTION

There are two aims in this study. The first is whether homologous superoxide dismutase (SOD) suppresses the ischaemic paw oedema of the same species, and the second is to establish a simple *in vivo* screening method for SOD-like compounds. Rat erythrocyte Cu,Zn-SOD, when injected intraperitoneally (i.p.), was not suppressive on rat models of carrageenan paw oedema, adjuvant arthritis, adriamycin-induced and ischaemic paw oedemata according to Michelson *et al.*[1] These workers predicted that human SOD may not be clinically effective from experiments with rats. It is possible that very little exogenous homologous SOD might not have any effect on the abundance of endogenous SOD. Some recognition is obviously required for the fixation of SOD to the target cell. When bovine erythrocyte SOD was encapsulated in liposomes various positive clinical results were obtained by intravenous (i.v.) injections.[2] We adopted i.v. dosing because i.p. dosing is not clinically useful and because of the risks of false suppression due to the counter irritancy. As it is possible that the rat is an exceptional case, we applied various sources of SODs including a homologous one on 3 differnt species. Our second aim was fulfilled by the characterization of ischaemic paw oedema of mice. Various drugs such as the antioxidants, cyclooxygenase and lipoxygenase inhibitors were examined together with SOD in this model. Drugs which reduce the ischaemic paw oedema may possibly ameliorate ischaemic damage in heart, brain and intestine.

MATERIALS AND METHODS

Animals

Male ddY (27–33 g) mice, male Sprague-Dawley rats (230–270 g) and male Hartley guinea pigs (270–330 g) were obtained from Sizuoka Agr. Coop. Assoc. Aged animals were also used for extraction of erythrocyte SOD.

Preparation of SOD

The animals were scissored at the front neck and the blood was collected in a beaker containing heparinized saline. Half diluted blood was passed through cotton gauzes to eliminate hairs and debris. Preparation of SOD was carried out according to the method of McCord and Fridovich.[3] Eluted fractions from DE32 column were checked for their SOD activities by the nitrite method[4] and the unit of final preparations was determined by the cytochrome c method.[3]

Electrophoresis

The method of Beauchamp and Fridovich[5] was applied for detecting the purity of SOD. Polyacrylamide gel electrophoresis (PAGE) was performed on a gel sheet of 80 × 60 × 1 mm. Illumination of the sheet containing nitroblue tetrazolium (NBT) and riboflavin was stopped when suitable contrast of formazan for detecting SOD activity was obtained. Coomassie Brilliant Blue was used for protein staining.

Sigma bovine Cu,Zn-SOD (Lot B) had 4 protein bands (0.38, 0.43, faint 0.47 and very faint 0.65 in terms of relative mobility from anodes) in which only the first 2 bands showed SOD activities. Another 4 lots tested also possessed proteins of mobility 0.31 and 0.53, but SOD activity was always on two bands of 0.38 and 0.42. It is well known that even a highly purified preparation of Cu,Zn-SOD showed 2 active forms of different electrophoretic mobility.[6,7] Therefore, we chose lot B for *in vivo* tests without further purification. For Sigma human Cu,Zn-SOD, we chose lot A which showed two SOD active bands of mobility 0.49 and 0.53. There was a faint band without SOD activity at 0.36, but not at 0.25 and 0.32 found in lot B and C. *Serratia* Mn-SOD had a single SOD band at 0.40. *Bacillus* Mn-SOD showed SOD positive 0.71 major band and very little SOD negative bands at 0.40 and 0.54.

Three preparations of mouse Cu,Zn-SOD exhibited only one protein band (0.34) possessing SOD activity. NBT staining demonstrated the existence of another weak SOD active band at 0.36. Rat Cu,Zn-SOD preparations showed a single active band at 0.46 tailing a little on the cationic side. Guinea pig Cu,Zn-SOD was obtained in low yields which had 2 SOD positive bands at 0.61 and 0.65.

Assay of Ischaemic Paw Oedema

Always the same commercial rubber ring (1 × 1 mm, d = 42 mm) was used for making ischaemia in 3 different animals. For mice, saline or drug solution (0.5 ml) was injected intravenously (i.v. by the tail) or intraperitoneally (i.p.) and the right hind leg was bound 12 times with a rubber ring at just above the articulation. The mouse was placed in a plastic cylinder device being picked up by the right leg from the slit. Up to 20 mice could be tested by two persons in less than 1 hr. The rubber ring was scissored off after 20 min using the same device and immediately the paw thickness

was measured with Citizen Thickness Gauge KG-1 (Citizen Watch Co., Tokyo). The swollen paw was generally measured at 20 min after the recirculation. One test group consisted of 4 mice and 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 for a dose of a drug in different days. Thirty percent suppressive dose (ID_{30}) was adopted to represent the potency of sample because the maximum suppression of paw oedema was nearly 60%. Water insoluble drugs were dissolved in *dimethylsulphoxide (DMSO)* making the final concentration of this solvent as 5% with saline. Only slight increase in paw swelling resulted with this concentration of DMSO. For fixing the rats, a more solid plastic device was applied. Two rubber rings were bound 12 times. After 45 min ischaemia, the rubbers were scissored off and the paw volume measured with water replacement apparatus (MK-500 Digital Volume Meter, Muromachi Kikai Co., Tokyo). Drug solution (1.0 ml) was injected (i.v. at the tail) juts before ischaemia. Four rats were used for the drug group and 5 rats for control.

Guinea pig ischaemia was induced by 12 times binding of two rubber rings using the same device for rats. One hr ischaemia-1 hr recirculation system was adopted after the time-course study. Drug solution (1.0 ml) was injected i.v. into the vein exposed by cutting off a part of the skin of the fore paw. Four animals (5 for control) represented a group.

Chemicals

Bovine blood (3,300 U/mg protein, lot B) and human blood (2,580 U/mg protein, lot A) Cu,Zn-SOD, cytochrome c (type III, from a horse heart), adenosine triphosphate-

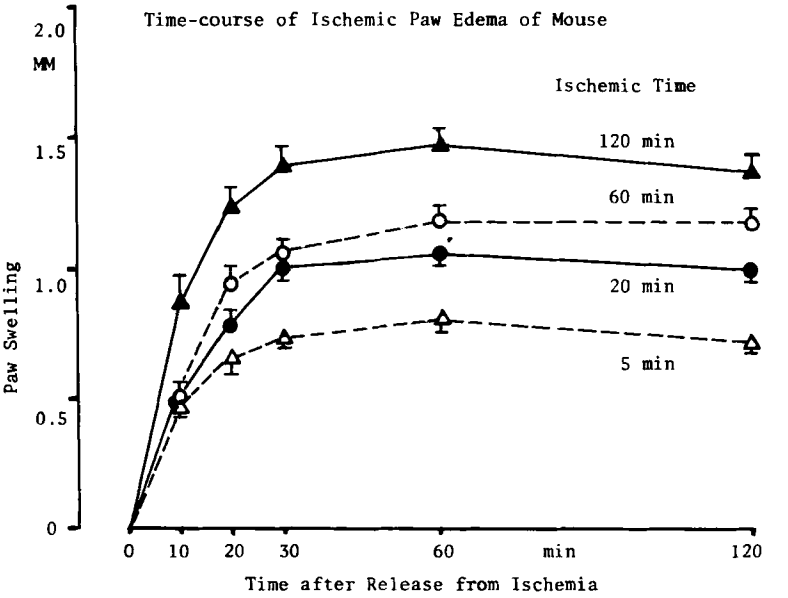

FIGURE 1 Time-course of Ischaemic Paw Oedema of Mouse (mean ± S.E. n = 12, normal paw thickness = 2.10 ± 0.06 mm).

TABLE I
Non-suppressive drugs on ischaemic paw oedema of mouse

Drugs (i.v.)	Inhibition by 10 mg/kg	Drugs (i.v.)	Inhibition by 10 mg/kg
Heat inactivated bovine Cu, Zn-SOD	5 ± 2	Indomethacin	6 ± 2
Bovine liver catalase	8 ± 1	Diclofenac sodium	13 ± 3
Glutathione peroxidase	11 ± 1	Ibuprofen	11 ± 2
Mannitol	13 ± 2	Benzydamine	13 ± 2
Dimethylsulphoxide	12 ± 1	Tiaramide	6 ± 4
Phenidone	6 ± 3	Reduced glutathione	20 ± 2
NDGA	6 ± 1	Dithiothreitol	18 ± 2
Quinacrine	11 ± 2	D-Penicillamine	9 ± 2
Mepyramine	2 ± 2	L-ascorbic acid-Na	11 ± 2
Methysergide	9 ± 3	α-Tocopherol	7 ± 2
Hypoxanthine	−11 ± 3	Nifedipine	15 ± 2
Adenosine	−3 ± 1	Diltiazem	11 ± 1
ATP − 2Na	−1 ± 2	Verapamil*	15 ± 2
		Nafazatrom	6 ± 2

*3.2 mg/kg, because 10 mg/kg was lethal.

2Na: ATP) and nordihydroguaiaretic acid (NDGA) were the products of Sigma Co. *Serratia marcescens* Mn-SOD (12,000 U/mg protein) and *Bacillus stearothermophilus* Mn-SOD (8,200 U/mg protein) were purchased from Wako Pure Chem. Co. (Osaka) and Unitika Co. (Kyoto), respectively. Liposomal bovine blood Cu,Zn-SOD (3,600 U/mg protein SOD in 6 mg solid) were kindly offered from Dr. Michelson (Paris) through Dr. Niwa (Tosashimizu, Japan). Bovine liver catalase (1,500 U/mg solid) and glutathione peroxidase (type III, 50 U/mg solid) were products of Tokyo Kasei Co. (Tokyo) and Toyobo Co. (Osaka). Dexamethasone (Decadron®, 3.3 mg/-ml sodium phosphate, Japan-Merck Co.) was for human therapeutic use. Trasylol® (aprotinin, a proteinase inhibitor from bovine lung, 2×10^4 KIE U/5 ml vial, Bayer-Takeda Co.) was also an injectable solution. Crystals of mepyramine · bimalate (Sandoz Co.) were dissolved in saline. A fine suspension of hypoxanthine (Teijin Co., Osaka) was prepared with sonication. Indomethacin (August Brandes Co.) was dissolved in a small volume of 1N-NaOH before saline addition and the pH was finally adjusted to 7.0 with 0.1 N-HCl. Diclofenac sodium, ibuprofen, diltiazem, verapamil and nafazatrom were synthesized in this laboratory for experimental use and dissolved in dimethylsulphoxide (DMSO) followed with addition of saline to make a final 5% solution of DMSO. Benzydamine and tiaramide were also synthesized in this laboratory and dissolved in saline. Other drugs of possibly pure grade were purchased from Nakarai Chem. Co. (Kyoto) and dissolved in saline or 5% DMSO-saline solution.

RESULTS

Time-course of Oedema Formation

Paw swelling of mice was measured for up to 120 min. From Figure 1, 20 min ischaemia-20 min recirculation system was decided to test the effect of a drug. A

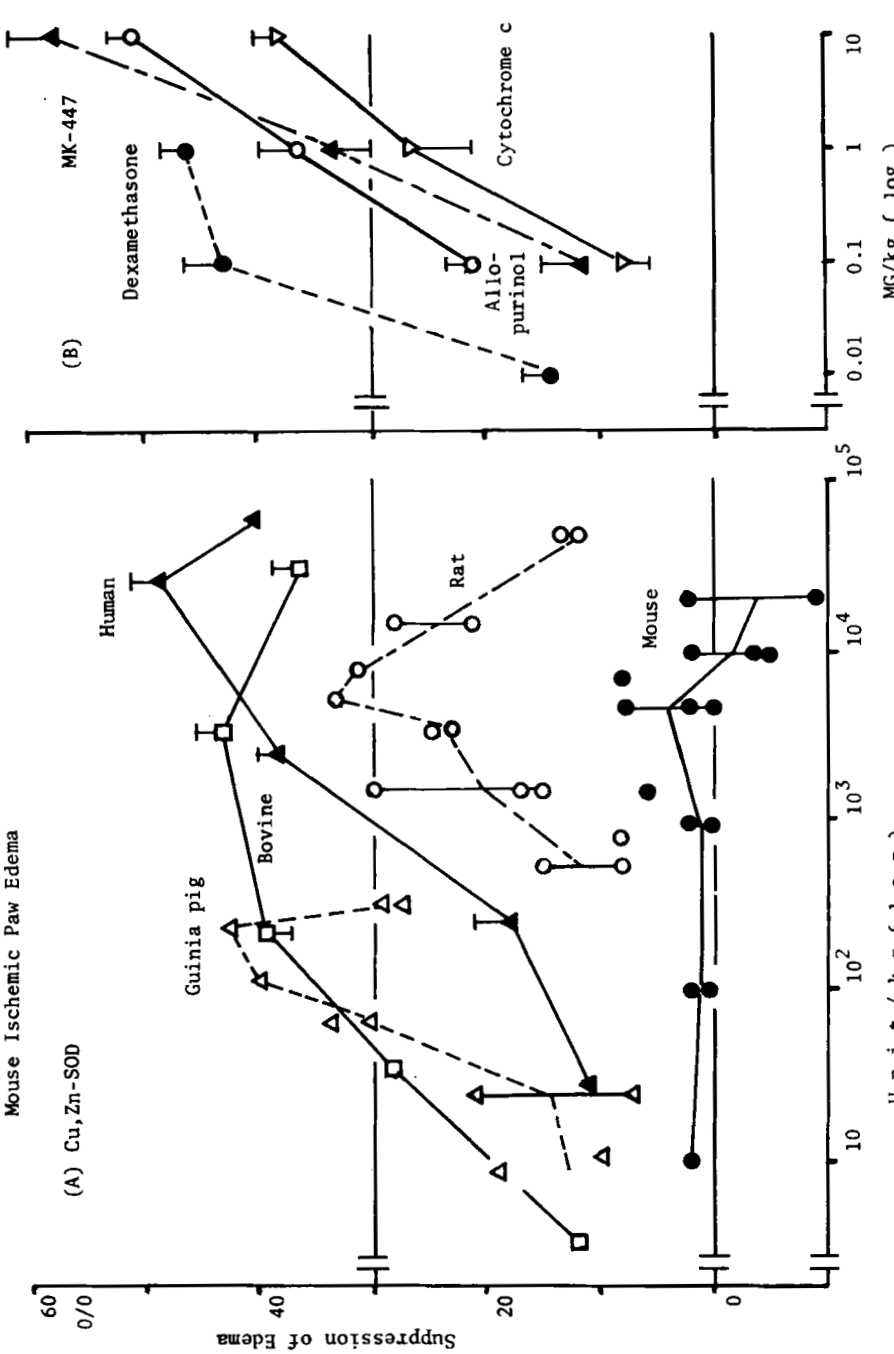

FIGURE 2 Suppression of Ischaemic Paw Oedema in Mouse (20 min Ischaemic-20 min Recirculation, mean of 4 mice or mean ± S.E. with 12 mice).

TABLE II
Suppression by SOD of ischaemic paw oedema in mouse (20 min ischaemia-20 min recirculation)

SOD (n = 12)	ID_{30} U/kg (protein/kg)	
	i.v.	i.p.
Serratia Mn-SOD	60 (0.005)	36,00 (3)
Bovine Cu, Zn-SOD	64 (0.02)	max.26% by 10 mg/kg
Guinea pig Cu, Zn-SOD	75 (0.04–0.09)*	N.D.
Human Cu, Zn-SOD	100 (0.04)	75,000 (30)
Bacillus Mn-SOD	306 (0.04)	N.D.
Rat Cu, Zn-SOD	400 (0.08–0.12)*	N.D.
Mouse Cu, Zn-SOD	no suppression up to 20,000 U/kg	N.D.
Liposomal bovine Cu, Zn-SOD	2,520 (0.7)	4,300 (1.2)

N.D.: Not determined,
All Cu, Zn-SOD were from erythrocytes.
*; As 3 preparations were used, exact mg/kg not obtained.

normal paw was about 2.1 mm thick and a 0.7–0.8 mm increase resulted. No significant difference of paw swelling was observed from 10 to 14 rubber ring bindings. Time-course of rat paw swelling by 11-times-bindings of two rubber rings was followed until 120 min after various duration of ischaemia. Standard assay condition was taken as 45 min–30 min recirculation which resulted in a 0.7–0.9 ml gain of paw volume. Normal paw volume was about 1.7 ml. Bindings of 9–12 times gave nearly the same swellings. After similar swellings were obtained by 11 times bindings of two rubbers the standard system for guinea pigs was decided as 60 min ischaemia-60 min recirculation (0.6–0.7 ml paw volume increase). No significant difference of increased paw volume was observed between 10 to 13 times rubber bindings.

Suppression of Mouse Oedema by I.V. SOD and Other Drugs

Bovine and guinea pig Cu,Zn-SOD (i.v.) exhibited similar low ID_{30} (Figure 2A and Table 2), but the former had a broader range of suppressive potency of over 30% than the latter. Human and rat Cu,Zn-SOD had higher ID_{30} and rat SOD showed a very narrow range of suppressive dose. *Serratia* Mn-SOD had a broad effective range (70-1.2 × 10^5 U/kg over 30%), but *Bacillus* Mn-SOD had a rather narrow range (300-2 × 10^3 U/kg over 30%). Surprisingly mouse Cu,Zn-SOD had no suppression. Liposomal bovine Cu,Zn-SOD weakly suppressed the oedema when injected i.v. (2.9 × 10^4 U/kg = 8 mg as SOD/kg).

Allopurinol suppressed the oedema (ID_{30} = 0.3 mg/kg) suggesting that O_2^- generated by xanthine oxidase (XOD) is responsible for this model (Figure 2B). MK-447 also proved to be suppressive. A hydroxy-like radical can be produced when the strong inflammatory PGG_2 is converted to less active PGH_2. This radical was reported to be eliminated by MK-447,[8] though there is an objection to this theory.[9] Dexamethasone suppressed when injected 3 hrs in advance (ID_{30} = 0.03 mg/kg) and not when injected just before ischaemia. ID_{30} of cytochrome c (O_2^- scavenger) was 5.0 mg/kg and that of dimethylthiourea (DMTU; ·OH and H_2O_2 scavenger) was 28 mg/kg. As reported in rats,[10] Trasylol® (1.2 × 10^5 KIE unit/kg) decreased 30% of oedema of mice. Conversion of xanthine dehydrogenase (XDH, inactive type in

normal tissue) to XOD (O_2^- generating type in ischaemia) might be blocked by this proteinase inhibitor.

NON-SUPPRESSIVE DRUGS ON MOUSE OEDEMA BY I.V. DOSE

Non-suppressive drugs (i.v.) are listed in Table 1. Heat inactivated (100°C, 30 min) bovine Cu,Zn-SOD had no suppressive effect. Mannitol and DMSO also showed no suppression. However, their ·OH scavenging capacities are not potent and SOD plus catalase was effective indicating that we could not exclude the possible participation of ·OH. Leukotriene synthesis inhibitors (phenidone and NDGA), cyclooxygenase inhibitors (indomethacin, diclofenac sodium and ibuprofen) and phospholipase A_2 inhibitor (quinacrine) had no effect, so that arachidonic acid metabolites were not involved in our model. Non-acidic anti-inflammatory drugs (benzydamine and tiaramide) were also not suppressive. Neither histamine nor serotonin was the mediator, since mepyramine and methysergide could not be demonstrated to be suppressive. Calcium channel blockers (nifedipine, diltiazem and verapamil) and an anti-thrombotic agent (nafazatrom) were tested and found to be negative. SH-containing compounds were insignificantly effective. Ascorbic acid and α-tocopherol were also negative. Hypoxanthine (a substrate for XOD) and its precursors (adenosine and ATP) marginally enhanced the oedema formation, suggesting that hypoxanthine was already sufficiently accumulated in the paw after the ischaemic state of 20 min.

Suppression of Mouse Oedema by I.V. Dose of SOD plus Catalase

High doses of SODs were tested with or without catalase (1,500 U/kg) injected as a mixture with SOD. A decrease in the suppression by higher doses of rat and bovine Cu,Zn-SOD was partially prevented with catalase (Figure 3). This could be interpreted as the result of removal of H_2O_2 generated by a high dose of Cu,Zn-SOD since H_2O_2 could inactivate the enzyme. Nevertheless suppression by a high dose of Mn-SOD also recovered. As Mn-SOD cannot be inactivated by H_2O_2, another mechanism such as inhibition of ·OH formation by catalase must be implicated.

Suppression of Mouse Oedema by I.P. Dose of SOD

Some SODs were also tested i.p.. Human Cu,Zn-SOD was required 750 times more in the case of an i.p. dose (about 30 mg/kg versus 0.04 mg/kg) for 30% suppression (Table 2). Free bovine Cu,Zn-SOD (i.p.) showed only 26% suppression even by 10 mg/kg, yet liposomal preparations containing the same dose of the same SOD were very effective by i.p. injection (ID_{30} = 1.2 mg/kg = 4,300 U/kg).

Suppression of Rat Oedema by I.V. Doses of SODs

The most potent was guinea pig SOD (ID_{30} = 30 U/kg to suppress rat oedema by i.v. dosing) followed by mouse SOD (ID_{30} = 70 U/kg) (Figure 4A). Homologous rat Cu,Zn-SOD was less suppressive (ID_{30} = 800 U/kg), yet still more effective than bovine Cu,Zn-SOD (ID_{30} = 9,000 U/kg). All SODs showed bell-shape suppressions. Allopurinol (i.v. just before ischaemia) and dexamethasone (i.v. injected 3 hrs before ischaemia) also suppressed rat oedema.

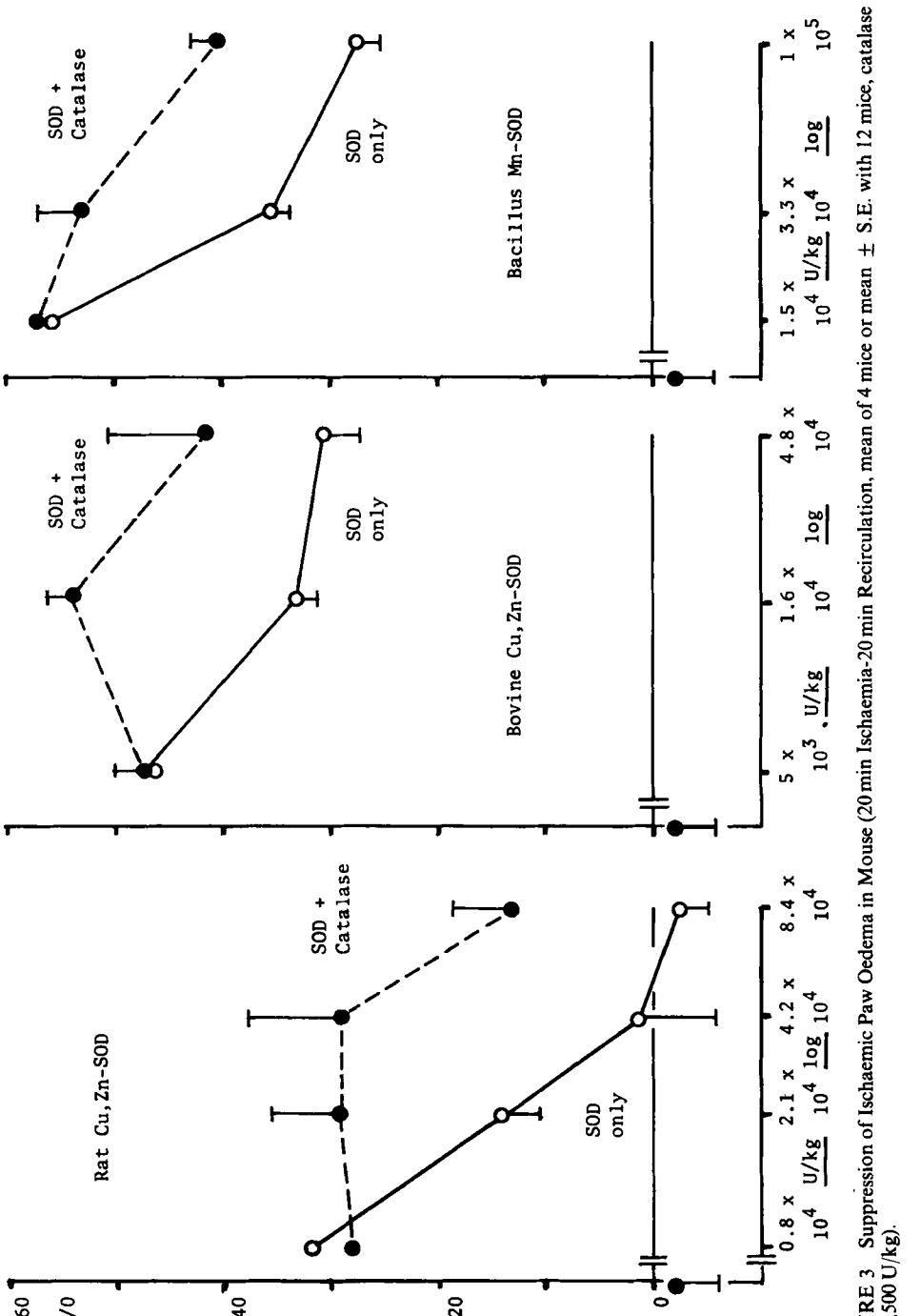

FIGURE 3 Suppression of Ischaemic Paw Oedema in Mouse (20 min Ischaemia-20 min Recirculation, mean of 4 mice or mean ± S.E. with 12 mice, catalase was 1,500 U/kg).

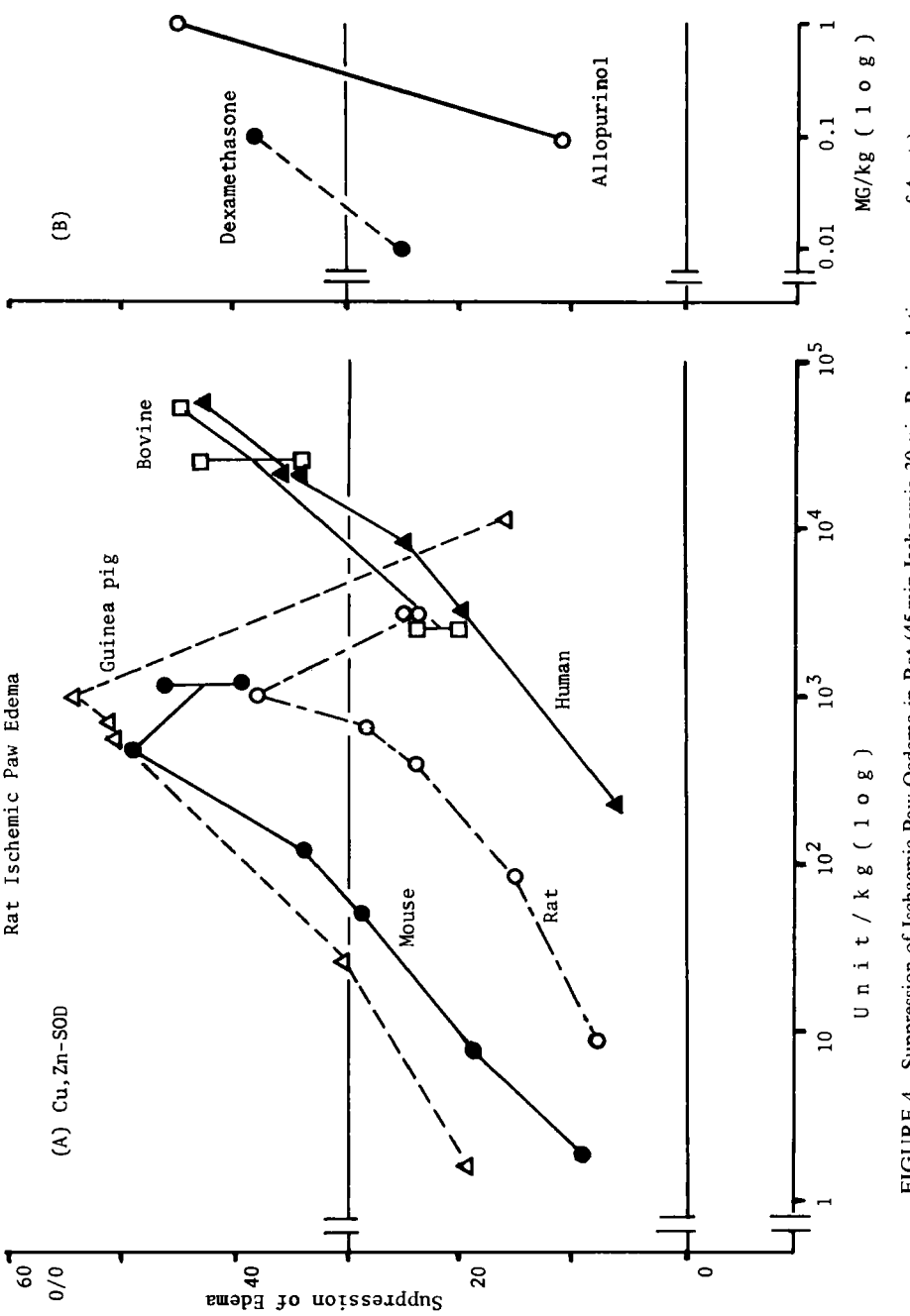

FIGURE 4 Suppression of Ischaemic Paw Oedema in Rat (45 min Ischaemia-30 min Recirculation, mean of 4 rats).

Suppression of Guinea Pig Oedema by I.V. Doses of SODs

Homologous guinea pig SOD was the most potent (ID_{30} = 30 U/kg), but with a very weak narrow range of effect. Rat SOD (40 U/kg) and mouse SOD (350 U/kg) followed a similar pattern. Bovine Cu,Zn-SOD was very weak (2,000 U/kg). Allopurinols and dexamethasone (3 hrs in advance) were suppressive.

Duration of the Effect of SOD on Mice Oedema

Free bovine Cu,Zn-SOD (i.v.) can be eliminated very rapidly in urine ($t_{1/2}$ = 6 min) and indeed a rapid loss of its potency (Figure 6) could be observed. Liposomal bovine Cu,Zn-SOD, in contrast, was suppressive even after 3 hrs (i.v. and i.p.). Dexamethasone suppressed only 10% and 16% when injected just before ischaemia and 60 min before recirculation. Nifedipine (i.v. 10 mg/kg) tested up to 180 min proved always to be non-suppressive. In our model of ischaemia it is useful also to know the duration time of the drug effect.

DISCUSSION

Mouse SOD (i.v.) was not suppressive on mice, but rat SOD and guinea pig SOD suppressed the ischaemic oedema of the same species. Distribution of SOD is restricted to intracellular spaces, so that even a small amount of exogenous homologous SOD could change the radical state in blood vessels. The order of potency was: bovine $\geqq$ guinea pig $\gg$ human > rat $\gg$ mouse (no suppression) on mouse oedema; guinea pig > mouse > rat > bovine $\geqq$ human on rat oedema; and guinea pig > rat > mouse > human $\geqq$ bovine on guinea pig oedema. Our preliminary experiments with carrageenan paw oedema of rats indicated that these SODs (i.v.) suppressed the paw swelling at 3 hr in the following order: guinea pig > mouse > rat > human $\geqq$ bovine (data not shown). The narrow effective range of rat and guinea pig Cu,Zn-SODs can be easily overlooked unless various doses are tested. Natural human and bovine Cu,Zn-SODs have a rather broad effective range. If this is true also in humans, a therapeutic dose can be easily established for bovine and human Cu,Zn-SOD. In the case that a combination with catalase is clinically useful to widen the effective range, Mn-SODs and guinea pig Cu,Zn-SOD are also worth considering after blocking of their antigenicity. Application of allopurinol and its analogues against ischaemic oedema or disfunctions has been considered, although they have been reported to be toxic.[11,12] It was interesting that dexamethasone suppressed the paw oedema of these animals only when injected more than 3 hrs in advance. This suggested the participation of protein(s) which inhibit the vascular permeability. We have already studied such a protein named "vasoregulin" using serotonin-, histamine- and bradykinin-induced paw oedema models.[13,14] A similar protein "vasocortin" was reported later by Carnuccio *et al*,[15] using dextran-induced paw oedema of rats. Our conclusion was that both endogenous and exogenous glucorticoids are perpetually synthesizing vasoregulin (vascular permeability inhibitory protein) which is inactivated by O_2^-.[16] MK-447 showed protection on ischaemic oedema of mice. Antioxidants, scavengers as well as this type of compound, are the targets to be modified and to be tested in our model of mice for creating a new drug. Lack of suppression by cyclooxygenase inhibitors such as indomethacin in this model, offer a simple *in vivo* method to characterise new types of anti-inflammatory or anti-ischaemic drugs.

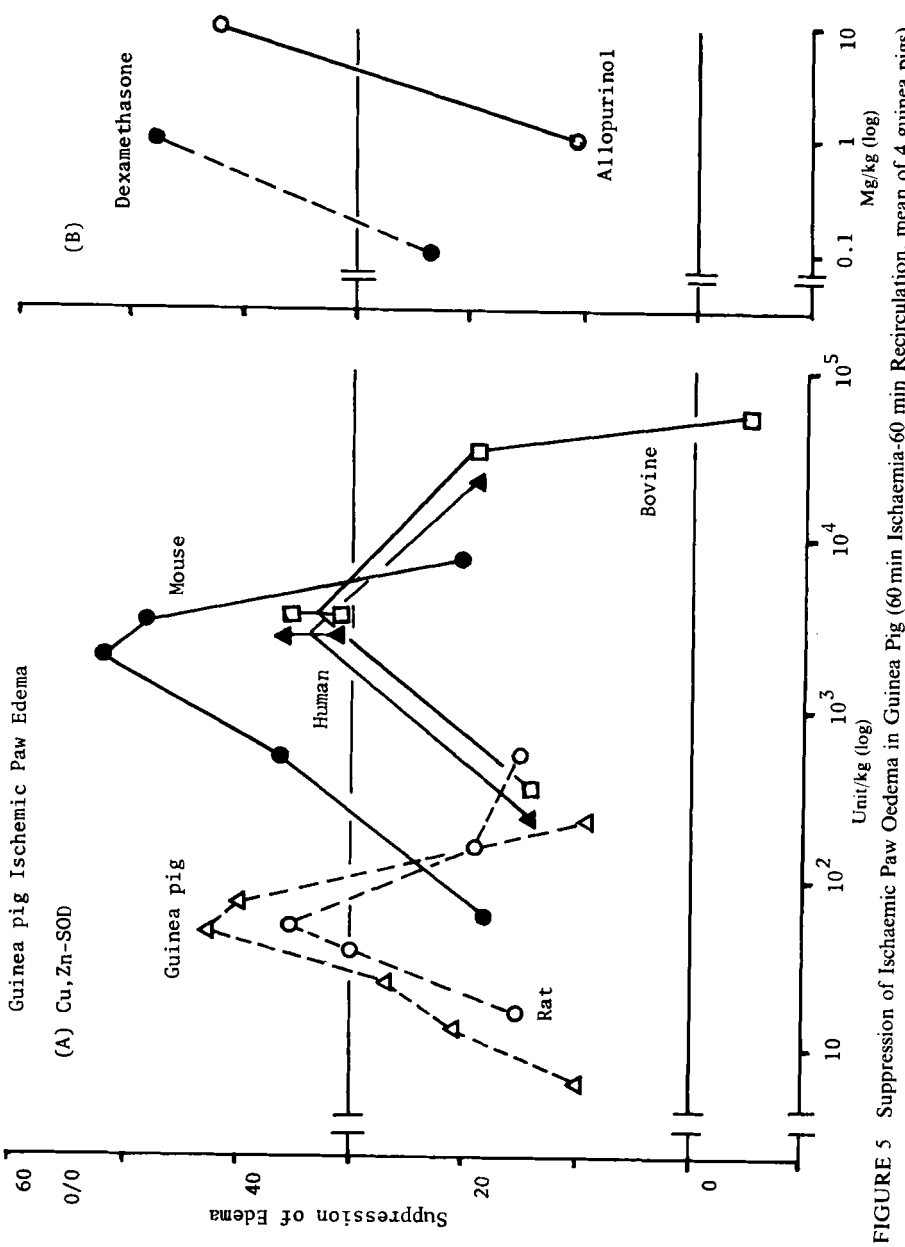

FIGURE 5 Suppression of Ischaemic Paw Oedema in Guinea Pig (60 min Ischaemia-60 min Recirculation, mean of 4 guinea pigs).

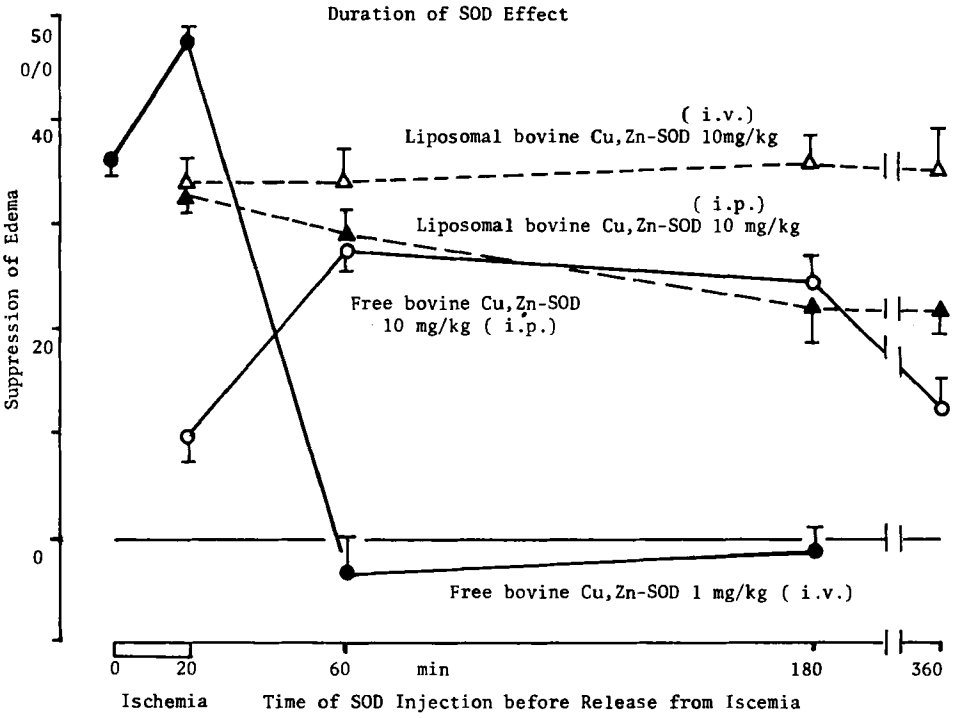

FIGURE 6 Duration of Suppressive Effect of SOD on Ischaemic Paw Oedema of Mouse (mean ± S.E. with 12 mice).

References

1. Michelson Somiya, A.M., Puget, K. and Jadot, G. *Free Rad. Res. Comms.*, **2,** 43–56, (1986).
2. Niwa, Y., Somiya, K., Michelson, A.M. and Puget, K. *Free Rad. Res. Comms.*, **1,** 137–153, (1985).
3. McCord, J.M. and Fridovich, I. *J. Biol. Chem.*, **244,** 6049–6055, (1969).
4. Ōyanagui, Y. *Anal. Biochem.*, **142,** 290–296, (1984).
5. Beauchamp, C. and Fridovich, I. *Anal. Biochem.*, **44,** 276–287, (1971).
6. Civalleri, L., Pini, C., Rigo, A., Calabrese, L. and Rotilio, G. *Mol. Cell. Biochem.*, **47,** 3–9, (1982).
7. Marelli, I., Ciriolo, M.R. and Rotilio, G. *Biochem. Biophys. Res. Commun.*, **117,** 677–681, (1983).
8. Kuehl, F.A., Jr., Humes, J.L., Egan, R.W., Ham, E.A., Beveridge, G.C. and Van Arman, C.G. *Nature,* **265**, 170–172, (1977).
9. Harada, Y., Tanaka, K. and Katori, M. *Prostaglandins,* **28,** 367–381, (1984).
10. Eigler, F.W., Stock, W. und Höfner, I. *Z. ges. exp. Med.*, **151**, 55–63, (1969).
11. Singer, J.Z. and Wallace, S.L. *Arthritis and Rheumatism,* **29,** 82–87, (1986).
12. Suzuki, Y. and Sudo, J. *Japan J. Pharmacol.*, **45,** 271–279, (1987).
13. Ōyanagui, Y. *Biochem. Pharmacol.*, **30,** 1791–1798, (1981).
14. Ōyanagui, Y. *Inflammation,* **7,** 81–89, (1983).
15. Carnuccio, R., DiRosa, M., Guerrasio, B., Iuvone, T. and Sautebin, L. *Br. J. Pharmacol.*, **90,** 443–448, (1987).
16. Ōyanagui, Y. and Suzuki, S. *Agents and Actions,* **17,** 270–277, (1985).

Accepted by Prof. J.V. Bannister