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ANTI-INFLAMMATORY ACTIVITY OF SUPEROXIDE DISMUTASES: INHIBITION OF CARRAGEENAN INDUCED EDEMA IN RATS

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Eighteen different superoxide dismutases from procaryote, plant, fish, bird and mammalian species have been tested for anti-inflammatory activity in the rat paw pad carrageenan-induced inflammation model. Very large differences in activity are observed. Homologous rat Cu-SOD is not active and indeed shows slight **pro**-inflammatory activity. The different SODs have different iso-electric values, different metals (Cu, Mn or Fe) at the active centre, different molecular weights and different circulation lifetimes. Biological activity is a function of amino acid sequence rather than of such secondary parameters.

Key words: Carrageenan edema, inflammation, superoxide dismutases, enzymotherapy

INTRODUCTION

As a result of the pioneering work of W. Huber and K.B. Menander-Huber on the clinical application of bovine copper superoxide dismutase, begun several years before identification of the enzymic activity of the protein by McCord and Fridovich[1], the anti-inflammatory properties of this natural biological drug have been extensively studied. Although the substrate specificity and catalytic mechanisms of the different Cu, Mn or Fe containing superoxide dismutases are now established and known in detail, the anti-inflammatory activity in mice and men is but poorly understood. As part of an attempt to increase this understanding we present in this (and subsequent) report(s) studies on the anti-inflammatory activities of SODs containing different metals (Cu, Mn or Fe) at the active centre, different iso-electric points, different circulation lifetimes, and different molecular weights isolated from procaryote, plant, fish, bird and mammalian species using different inflammation models in the rat.

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Carrageenan-induced inflammation in the rat paw[5] is a simple model which is widely used for pharmacological screening of anti-inflammatory drugs. The edema produced is limited to the primary phase of vasodilatation, plasmatic exudation and migration of polymorphonuclear neutrophils and monocytes. During the first two hours serotonin, histamine (at 1 hr) and kinines (at 2 hr) play a major role in a first phase, followed at 3 hr (kinines, beginning of prostaglandins) to 5 hr by a second prostaglandin phase. The easiest technique of measurement is simply to follow changes in volume. Given the nature of the test the results are relatively precise and at least in a qualitative sense allow comparison of the efficiency of different substances to inhibit swelling related to the first or second phase or both.

Various SODs isolated from different sources but all with the same enzymic activity have been compared with respect to their anti-inflammatory activity using this test.

MATERIAL AND METHODS

Carrageenan type S (Sigma) was prepared as a 1% solution in 0.9% NaCl at 37°C just before use. The right rear paw pad of the rat was cleaned with ether and 150 μl of the carrageenan injected. Measurements of volume were made immediately before injection, and at 1, 2, 3 and 5 hours later by immersing the paw in the cuvette of a water plethysmometer (Ugo Basile) with a Wheatstone Bridge. Variation of the resistance is directly proportional to variation of volume in the cuvette, that is to paw volume (Archimede's technique) and gives numerical values by which even small changes can be followed. The rats were male Wister AF-gnotoxenic with a weight of 300 ± 20 g. A total of 454 rats were randomised and divided into the different testing groups. For controls, 28 animals were injected 30 min before carrageenan treatment with 1 ml of 0.9% NaCl at 37°C. A second internal control (14 rats) was also used by injection of phenylbutazone (1 ml at 37°C) at 100 mg/kg again at 30 min before time zero. Similarly the SODs were administered in 1 ml at 37°C at 30 min before carrageenan. All injections (other than carrageenan) were intraperitoneal. Except where otherwise stated the SODs were examined at 33 μg/kg and a minimum of 10 animals was used for each value. To eliminate possible nycthemeral variations all batches were treated at 9.00 a.m. The use of an internal control with phenylbutazone validates the edema test each time and allows a comparison not only among different SODs but also with a reference anti-inflammatory drug. Another control, not discussed in detail, employed bovine and human albumin at 6.7 to 167 μg/kg (42 rats). No anti-inflammatory activity was observed.

Probabilities were calculated according to the Student t test for the comparisons of mean values of change in volume. To test homogeneity of the measures, variations were controlled by the technique F of Snedecor.

Superoxide dismutases were isolated and purified by established techniques[6] and were all single band proteins on gel electrophoresis. Isoenzymes were in general separated by chromatofocalisation. Specific activities of all Cu-SODs were approximately 3000-3300 NBT/riboflavin units/mg. Human Mn-SOD had a specific activity of 2400 units, the Fe-SODs and *E. coli* Mn-SOD had specific activities of 1500–1600 units/mg. Albumin coupled bovine Cu-SOD was prepared with purified human albumin.

Details of absorption at 260 nm and 280 nm with respect to protein concentration measured by fringe interferometry are given below.

Relationship between absorption and protein concentration determined by fringe interferometry

SOD	Absorbance ratio 280 nm/260 nm	At 1 mg/ml Absorption at 260 nm	280 nm
E. coli Fe-SOD	1.596	1.247	1.990
P. leiognathi Fe-SOD	1.696	1.179	2.000
E. coli Mn-SOD	1.829	0.960	1.756
Human Mn-SOD	1.620	1.562	2.530
Bovine Mn-SOD	1.918	1.314	2.520
Cabbage Cu-SOD	0.570	0.300	0.171
Leek Cu-SOD I	0.775	0.306	0.237
Leek Cu-SOD III	0.540	0.450	0.243
Horse Cu-SOD	0.366	0.333	0.122
Turkey Cu-SOD	0.740	0.436	0.323
Swordfish Cu-SOD	0.800	0.357	0.285
Human Cu-SOD	0.955	0.560	0.535
Rabbit Cu-SOD I	0.396	0.298	0.118
Rabbit Cu-SOD II	0.397	0.270	0.107
Rat Cu-SOD	0.632	0.280	0.177
Pig Cu-SOD	0.910	0.368	0.335
Yeast Cu-SOD	0.690	0.307	0.212
Bovine Cu-SOD Japanese	0.568	0.336	0.191
German	0.575	0.294	0.169
French	0.599	0.297	0.178

Typical results with details of a single SOD are as follows:

Male rats 300g

	Controls (n = 28)				*Phenylbutazone treated 100 mg/kg* (n = 14)				*Swordfish Cu-SOD treated 33μg/kg* (n = 10)			
	Hours after carrageenan injection.				Hours				Hours			
	1	2	3	5	1	2	3	5	1	2	3	5
Mean increase in plethysmometer reading	40.7	41.2	63.1	41.4	20.1	17.8	14.9	23.4	16.3	11.7	16.5	15.0
±	5.3	4.9	12.1	5.0	3.0	4.3	4.1	3.4	2.7	3.7	4.2	4.7
% change compared with controls	—	—	—	—	−50.6	−56.8	−76.4	−43.5	−60.0	−71.6	−73.8	−63.7
p <	—	—	—	—	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

RESULTS AND DISCUSSION

Plethysmometric measurements were made at 0, 1, 2, 3 and 5 hr after initiation of the carrageenan-induced inflammatory edema. To simplify presentation of the results an average value of the percentage change in volume compared with the control (without treatment) of the figures obtained at 1, 2, 3 and 5 hr covering both the early and the late phases is given. Each percentage change is the average of at least 10 rats for each

SOD, with 14 animals for the internal control with phenylbutazone which has very significant anti-inflammatory activity in both phases. The control group (carrageenan injection only) used to measure volume of the edema was composed of 28 rats.

As with phenylbutazone, in general no significant differences in efficacity were observed between the first serotonin phase and the prostaglandin phase. Exceptions to this are presented in Table I in which mean values of change in volume for 1 plus 2 hr are compared with those at 3 plus 5 hrs. It can be noted that bovine and human Cu-SODs at low dose are more effective during the serotonin phase than during the second prostaglandin period but that these differences tend to disappear at higher doses. This may be due to clearance rates, but in contrast pig Cu-SOD, leek Cu-SOD and *E. coli* Fe-SOD are more efficient during the second phase. Human Mn-SOD shows properties which suggest a slight anti-inflammatory activity at low dose for the first phase but at higher levels (33 μg/kg) a significant pro-inflammatory effect (i.e. increase in volume greater than that seen in untreated carrageenan injected animals) is seen during the second phase. These variations undoubtedly reflect differences in the biochemical situation (leading to superoxide production) in the different phases but also imply a non-identical bio-availability for SODs from divers sources.

Both *E. coli* Mn-SOD and swordfish Cu-SOD show exceptional anti-inflammatory activity during all four time periods with percentage reductions in volume of 81.7,

TABLE I
Percentage change in volume, at early and late phase

	1 + 2 h	p <	3 + 5 hr	p <
Bovine Cu-SOD				
6.7 μg/kg	−71.0	(0.001)	−29.0	(0.01)
33.3 μg/kg	−69.1	(0.001)	−47.2	(0.005)
Human Cu-SOD				
6.7 μg/kg	−50.4	(0.01)	−16.2	(NS)
33 μg/kg	−50.5	(0.01)	−23.0	(NS)
167 μg/kg	−62.6	(0.001)	−51.8	(0.001)
E. coli Fe-SOD				
33.3 μg/kg	−23.3	(NS)	−54.6	(0.001)
Pig Cu-SOD				
33 μg/kg	+2.6	(NS)	−35.8	(NS)
Leek Cu-SOD (pI 4.6)				
33 μg/kg	−7.2	(NS)	−51.3	(0.001)
Human Mn-SOD				
6.7 μg/kg	−41.1	(0.05)	−18.4	(NS)
33.3 μg/kg	+0.6	(NS)	+30.6	(NS)
Rat Cu-SOD				
6.7 μg/kg	+46.9	(0.05)	+14.1	(NS)
33.3 μg/kg	+48.2	(0.05)	+32.4	(0.1)
167 μg/kg	+5.1	(NS)	+1.7	(NS)
Phenylbutazone				
100 mg/kg	−53.7	(0.001)	−60.0	(0.001)

Probabilities (given in parentheses) are those for difference with untreated carrageenan injected controls.

79.9, 87.9 and 80.5% at 1, 2, 3 and 5 hr respectively for the Mn-SOD ($p < 0.001$) and 60, 71.6, 73.8 and 63.7% for the Cu-SOD ($p < 0.005$). The pro-inflammatory activity of rat Cu-SOD is evident mainly at the first hour and to a lesser extent at later periods with percentage **increases** of volume above the control edema of 70.0 ($p < 0.01$), 26.5, 30.5 and 34.3% ($p < 0.05$) at the respective time periods (at 33 μg/kg).

The widespread concept that much larger pharmaceutical quantities should be employed in animals to correspond to human clinical treatment is perhaps true for many chemical drugs, but not necessarily so for enzymotherapy. Whereas others have often used injections of SOD in rats at 10–50 mg/kg i.e. 200 to 1000 times a normal clinical dose, we have preferred (in the absence of any guidelines for pharmaceutical comparisons between animals and humans with respect to use of **enzymes**) to employ levels roughly equivalent per kg to those routinely clinically applied. The negative aspects of a large excess of SOD with respect to protection *in vivo* are well established[7] and dose levels in rats[8] which correspond to daily injections of several g of enzyme in humans are perhaps exagerated and could give rise to other mechanistic interpretations (and secondary effects). Massive injections, whether of SOD, denatured SOD, polyethylene glycol-conjugated SOD or even of albumin could possibly lead to depletion of circulating factors such as complement, necessary for the inflammatory response[9]. At the dose rates used in this work, neither human nor bovine albumin showed any anti-inflammatory activity. Use of denatured SOD was not attempted in view of the impossibility of prevention of renaturation *in vivo*, whether apo-enzyme or halo-enzyme was denatured.

Anti-inflammatory activity of Ficoll coupled SOD has been observed in the carrageenan model at 3 mg/kg injected intravenously. However bovine Cu-SOD at this level had no anti-inflammatory activity[10]. This may be an example of the negative effects of excessive SOD (about 100 to 1000 times that used in this study). Increase in amount of i.v. injected SOD nullifies the radioprotective effects seen at low doses[7]. Alternatively, differences in administration (i.v. compared with i.p.) may be implicated. The maximum SOD generally available via the circulation occurs 1 hr or longer after intraperitoneal injection, and thus 30 min later than carrageenan induction (since the enzymes were injected prior to onset). Since the half time of clearance (to the kidney) after i.v. injection of bovine Cu-SOD is about 6 min very much less enzyme is available systemically compared with i.p. administration. Ficoll-

TABLE II

Average values for 1, 2, 3 and 5 hrs plethysometric measures as a function of concentration (percentage change)

	6.7 μg/kg	33 μg/kg	167 μg/kg
Bovine Cu-SOD	−50.0	−58.2	−13.1 (NS)
Bovine Cu-SOD conjugated to human albumin (equivalent weight of SOD)	−45.3	−75.6	−44.3
Human Cu-SOD	−33.3	−36.8	−57.2
Human Mn-SOD	−29.8	+15.6 (NS)	+10.1 (NS)
Rat Cu-SOD	+30.5	+40.3	+3.4
	15 μg/kg	*33 μg/kg*	
E. coli Mn-SOD	−85.0	−82.5	

Except where marked non-significant (NS) probabilities for difference with carrageenan injected untreated controls range from $p < 0.05$ to 0.001.

TABLE III
Percentage changes in volume at 1, 2, 3 and 5 hours for *E. coli* Mn-SOD at 15 μg/kg and 33.3 μg/kg

	Percentage change at				Mean value of 1, 2, 3 & 5 hr
	1 hr	2 hr	3 hr	5 hr	
Phenylbutazone 100 mg/kg	−50.6	−56.8	−76.4	−43.6	−56.8
E. coli Mn-SOD 15 μg/kg	−83.0	−81.6	−90.9	−84.5	−85.0
33.3 μg/kg	−81.7	−79.9	−87.9	−80.5	−82.5

All values of $p < 0.001$

SOD may act (at the concentrations used) as a scavenger for circulating inflammation factors (other than O_2^-) rather than catalytically, particularly in view of its longer circulating life-time.

The dose response for averaged values of 1, 2, 3 and 5 hr measurements for a number of SODs is shown in Table II. A slight preference for a routine use of 33 μg/kg corresponding fairly closely with human dosage rates, can be seen (except for human Cu-SOD) since the effects whether anti-inflammatory or **pro**-inflammatory (rat Cu-SOD) tend to decrease at the highest (167 μg/kg) level. For these reasons, the results with respect to bovine Cu-SOD (no other SODs having been till now seriously examined) presented in this study in which 33 μg SOD/kg is used to evaluate different SODs, are not strictly comparable to those in most other reports. When high anti-inflammatory activity is shown, decreasing the dose rate by about half (33.3 μg/kg to 15 μg/kg) has no effect at any of the biochemical phases between 1 and 5 hr as shown in Table III for *E. coli* Mn-SOD. The level chosen thus appears to be suitable for detection of differences in biological properties of the various SODs.

The relative efficacities of anti-inflammatory behaviour in the carrageenan edema rat paw model for 18 different SODs at 33 μg/kg are shown in Table IV, in which mean values of 1, 2, 3 and 5 hr measurements for the percentage change in volume compared with non-treated carrageen-induced volume increment controls are presented. A number of conclusions may be drawn from these results.

1) Different superoxide dismutases do **not** have an identical anti-inflammatory activity. Indeed in the rat carrageenan-edema model extremely large differences are observed. A highly active group contains *E. coli* Mn-SOD, swordfish, rabbit, and bovine Cu-SODs all equivalent or superior to phenylbutazone at 100 mg/kg. A second class with moderate to poor activity is composed of horse, human, yeast, leek (less acidic isozyme), turkey (less basic isozyme) Cu-SODs and *E. coli* Fe-SOD. This is followed by SODs with essentially zero anti-inflammatory activity (more acidic leek Cu-SOD isozyme, pig Cu-SOD, more basic turkey Cu-SOD isozyme, cabbage Cu-SODs and *P. leiognathi* Fe-SOD). Indications of **pro**-inflammatory properties are seen in human Mn-SOD and with homologous rat Cu-SOD the effects are definitely pro-inflammatory (in this model) leading to an **increase** in inflammatory swelling.

2) Anti-inflammatory activity is **not** a function of the nature of the metal at the active centre of the enzyme.

3) Biological activity *in vivo* is **not** a direct function of the isoelectric point of the

TABLE IV
Percentage change in volume compared with untreated carrageenan induced increment. Average of values at 1, 2, 3 and 5 hr

33 μg/kg	pI		Average of 1, 2, 3 and 5 hr results	p <
	Isoelectric focalisation	Chromato focalisation	% change in volume compared with untreated edema control	
E. coli Mn-SOD	6.4	7.8	−82.5	0.001
Swordfish Cu-SOD	5.4	7.5	−67.3	0.005
Rabbit Cu-SOD	4.8	6.2	−58.8	0.01
Bovine Cu-SOD	5.0	5.3	−58.2	0.001
Horse Cu-SOD	5.9	6.8	−44.3	NS
E. coli Fe-SOD	4.4	—	−39.0	0.01
Human Cu-SOD	4.8	—	−36.7	0.05
Yeast Cu-SOD	4.5	—	−30.5	0.02
Leek Cu-SOD	4.6	4.9	−29.2	0.01
Turkey Cu-SOD	—	7.35	−29.0	0.02
Leek Cu-SOD	4.25	4.1	−17.0	NS
Pig Cu-SOD	6.2	7.6	−16.6	NS
Turkey Cu-SOD	—	7.65	−12.0	NS
Cabbage Cu-SOD	4.3	4.7	−11.7	NS
Cabbage Cu-SOD	4.6	5.3	−7.2	NS
P. leiognathi Fe-SOD	4.1	—	+1.3	NS
Human Mn-SOD	7.4	—	+15.6	NS
Rat Cu-SOD (homologous)	5.0	6.3	+40.3	0.05
Phenylbutazone 100 mg/k	—	—	−56.8	0.001

Probability values are for differences with carrageenan induced untreated volume change.

protein. In general, an acidic pI of 4.5 (isoelectric focalisation) or less is associated with poor or zero anti-inflammatory properties but a basic pI does not necessarily confer activity. The value, whether acidic or basic, of pI does not of course rigorously reflect the real surface charge of a globular protein, and differences are seen dependent on the technique used.

4) Anti-inflammatory efficiency is **not** a direct function of molecular weight and is not necessarily increased if the size of the SOD is augmented. Thus human Mn-SOD has a molecular weight of 80 000 and *E. coli* Mn-SOD 40 000 but the smaller enzyme is vastly more efficient than the larger. Increase in molecular weight of bovine Cu-SOD by covalent fixation to human albumin does not improve activity which is practically the same as that of the parent molecule (Table II).

5) The anti-inflammatory properties of a given Cu-SOD are **not** directly related to circulation life times. Thus circulation half lives after intraperitoneal injection of bovine, human and rat Cu-SODs and human Mn-SOD are 3 hr, 1.6 hr, 1.5 hr and 6.52 hr respectively[11,12,13]. With respect to intravenous injection the half lives (first exponential) of the three copper SODs are essentially identical (~6 min) whereas that of human Mn-SOD is about 6.45 hr, for *E. coli* Mn-SOD, 44 min and for turkey Cu-

SOD, 10–14 min. Peak circulating activity occurs from 1 to 3 hr after intraperitoneal injection[12]; 3 hr after intravenous administration the homologous (marked) rat enzyme is present in the plasma at a 10 fold higher level than the three heterologous SODs[13].

6) Differences in biological activity **cannot** be explained by differences in specific enzymatic activity since all the copper Cu-SODs have the same value, 3150 ± 150 units per mg of protein (measured by fringe interferometry) using the NBT/riboflavin technique[14] for activity estimation and all contain 2 g atoms of Cu per molecule of enzyme. *E. coli* Mn-SOD (with **one** metal catalytic centre) has a specific activity about half that of the Cu-SODs but is nevertheless the most active anti-inflammatory agent. The two Fe-SODs have a specific activity slightly less than *E. coli* Mn-SOD whereas human Mn-SOD (two active centres but double the molecular weight) is somewhat more active per mg of protein.

7) Anti-inflammatory activity is limited (at least in this model) to heterologous SOD; homologous SOD is not efficient. The activity is indirect and unrelated to maintenance of high extracellular or circulating levels. Given the amounts of total endogenous SOD per kilogram of rat, it is clear that at the doses used (33 μg/kg) intracellular penetration in a general sense cannot be postulated as a major factor.

8) Amino acid sequence and hence minor topological, physical, antigenic determinant or binding site changes are important in the definition of anti-inflammatory activity of a given SOD when used for a specific animal[15]. Iso-enzymes (identical metal centre) from a given species do not have identical activity though differences are not large.

CONCLUSIONS

The primitive ideology postulating removal of extracellular superoxide radicals (produced for example by activated neutrophils) by exogenous freely circulating SOD as an explanation for the anti-inflammatory activity of bovine Cu-SOD is insufficient. Indeed, pro-inflammatory aspects may also be manifested, presumably as a result of the pro-oxidant properties of SOD in certain situations[16–19] when the enzyme contributes to the continued oxidation of another molecule such as menadione[20], rifamycin SV[21] or linoleic and arachidonic acids[22] by removing superoxide radicals. Thus $AH_2 + O_2 \rightarrow AH\cdot + O_2^- + H^+$ is followed by $AH\cdot + O_2 \rightarrow A + O_2^- + H^+$ if the reverse of the first reaction is inhibited by enzymic dismutation of superoxide anions. It may be recalled that enzymic dismutation is first order but spontaneous dismutation of O_2^- is second order and at low concentrations is extremely slow[23]. It is clear that mechanistic revision of the biological activities (as opposed to the undisputed enzymic activity) of superoxide dismutases is now necessary[15].

With respect to the properties of bovine Cu-SOD discrepancies with other reports can be attributed to different times of injection, different quantities of SOD and different techniques of administration, i.v., i.m. or i.p. which change the overall pharmacokinetics. Since the carrageenan test gives an evaluation of rapid action only, comparison of anti-inflammatory properties of different SODs is further developed in studies using the adriamycin-induced edema model (5 day test) in the rat[24].

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