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PHARMACOKINETIC AND ANTI-INFLAMMATORY PROPERTIES IN THE RAT OF SUPEROXIDE DISMUTASES (Cu SODs and Mn SOD) FROM VARIOUS SPECIES

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Abstract—The comparison of the anti-inflammatory properties (carrageenan paw edema in the rat) of exogenous Cu SODs from various species and human Mn SOD administered at doses corresponding to clinical schedules, shows that human and bovine Cu SOD are fully active, whereas rat Cu SOD and Mn SOD are inefficient. This difference does not correspond to the similarity of pharmacokinetic properties (blood levels, subcellar location in kidney cortex) of the Cu proteins or to the increased circulating life time of Mn SOD. The pharmacological activity of exogenous SOD is limited to heterologous Cu SODs in the rat. A similar problem may occur in man. The true mechanism of action of Cu SOD remains to be elucidated.

The anti-inflammatory activity of bovine Cu superoxide dismutase was empirically observed more than fifteen years ago, although the biochemical significance of the protein was then unknown.

The subsequent definition of this enzyme as a superoxide dismutase [1] and the discovery of superoxide anion O_2^- released by activated phagocytes [2] as a mediator of inflammation [3, 4], led to the hypothesis that exogenous Cu SOD could be pharmacologically active by decreasing O_2^- extracellular levels [5].

This conception is essentially based upon *in vitro* experiments, whereas *in vivo* studies led to contradictory results. These discrepancies are probably due to the pharmacokinetic properties of the protein (dose, biovailability, cell penetration) [6]. In fact, the pharmacological properties of bovine Cu SOD* alone have been studied in animals or man whereas those of other dismutases, particularly Cu SOD from isologous origin, and human Mn SOD, remain to be proved. On the other hand, the mechanism of kidney fixation, which is the major phenomenon of *in vivo* metabolism of extracellular Cu SOD [7, 8] has not been studied further. The present studies were undertaken to evaluate in the rat the correlation between pharmacokinetic characteristics (blood levels, subcellular location of Cu SODs in kidney cortex) and anti-inflammatory activities of various copper and manganese dismutases.

MATERIAL AND METHODS

Superoxide dismutases. Human, bovine and rat Cu SODs were purified from erythrocytes according to the method of McCord and Fridovich [9], with a slight modification. The specific enzymatic activities determined according to the riboflavin NBT method [10] were respectively 2900, 3200 and 3050 U/mg. Human Mn SOD (2300 U/mg) was purified from liver according to the technique of McCord [11]. The purity of these preparations was tested by polyacrylamide gel electrofocalization.

Pharmacokinetic studies. Pharmacokinetic properties were studied in Charles River male rats weighing 350–400 g. The enzymes were administered either i.v. or i.p. (500 μg/kg, 0.5 ml). Blood samples were obtained at specified times according to the nature of the enzyme and the injection route. The assay of Cu SOD and Mn SOD as proteins was effected in plasma according to radioimmunological techniques already described [12, 13]. Briefly a human homologous system (iodinated human Cu SOD anti-human Cu SOD antiserum RS 7) was used for the assay of human Cu SOD. This system is very species specific since RS 7 antiserum does not exhibit any cross reaction with endogenous rat Cu SOD and takes into account the exogenous protein alone. Likewise, the homologous rat system which was used does not evidence any cross reaction with human Cu SOD and allows the specific assay for rat endogenous plasma Cu SOD.

* Abbreviations. SOD, superoxide dismutase; Cu-SOD, copper containing superoxide dismutase; Mn-SOD, manganese containing enzyme; NBT, nitroblue tetrazolium; EDTA, ethylene diamine tetra-acetic acid; i.v., intravenous; TES, Tris, EDTA, saccharose buffer; PMN, polymorphonuclear leucocyte.

A similar homologous human system allowed the assay of Mn SOD but antihuman anti Mn SOD antiserum RS 3 shows a complete cross reaction with rat tissue enzyme and thus measures both endogenous and exogenous enzymes. The results are expressed as ng enzyme per ml of plasma.

Subcellular location of exogenous and endogenous Cu SODs in the cortex of rat kidneys. Male rats Charles River (350–400 g) were anaesthetized with Nembutal and injected with a tracer dose of rat, bovine or human iodinated Cu SOD which was diluted or not in a pharmacological dose of enzyme (250 μg). The tracers were obtained as described in [12].

After a period of 30 min, corresponding to the maximum of fixation, the animal was bled by intraortic punction, and the kidneys were excised. All procedures were then effected at 4°. After washings of kidneys in 15 mM Tris, 0.025 M Na_2 EDTA, 0.33 M saccharose pH 7.4 buffer (TES), the cortical area was excised, weighed and homogenized in TES buffer (1 g/5 ml) with a Dounce homogenizer. A filtration was effected with a nylon sieve, and the homogenate was centrifuged 10 min at 850 *g*. The supernatant was collected and centrifuged 3 min at 9000 *g*.

Three separate layers, brown bottom (A), yellow middle (B), and clear upper (C) are obtained. Three crude fractions, heavy lysozomes plus mitochondria (A), mitochondria plus light lysozomes (B) and brush border membranes (C), are carefully separated by suspending the three layers in TES buffer (500 μl). These fractions are rinsed by adding TES buffer (5 ml) and centrifuging 3 min at 9000 *g*. This operation is repeated twice.

An isopycnic centrifugation (30–60% saccharose gradient) of fraction A is carried out during 210 min at 10^5 *g* (SW 28 Kontron rotor). The gradient is divided in fractions (1 ml) which are analysed for radioactivity and enzymatic analysis. Bovine and rat Cu SODs are assayed as previously described [11]. Cytochrome oxidase is assayed according to [14] and acid phosphatase according to [15].

Anti-inflammatory activity testing. The anti-inflammatory properties of bovine, human and rat Cu SODs and human Mn SOD were evaluated by the measurement of inhibition of carrageenan paw swelling in male rats (390–450 g). Purified enzymes were injected i.p. 30 min prior to administration of 0.05 ml of carregeenan in saline into the paw. Each treated group received 500, 100 and 20 riboflavin units/kg, corresponding to 167, 33.3 and 6.6 μg/kg Cu SOD, and 217, 43.5 and 8.7 μg/kg Mn SOD. Plethysometric measurements were carried out 1, 2, 3 and 5 hr after injection of carrageenan. The results are expressed as the per cent variation of the mean of plethysmometric measurements for each treated group compared to the mean in a control saline group at the same time.

RESULTS

Endogenous SOD levels. Rat plasma Cu SOD levels ($N = 10$, $m = 248.11$ ng/ml, $sm = 29.06$) are easily assayed whereas immunoreactive Mn SOD appears to be undetectable in contrast with humans [13].

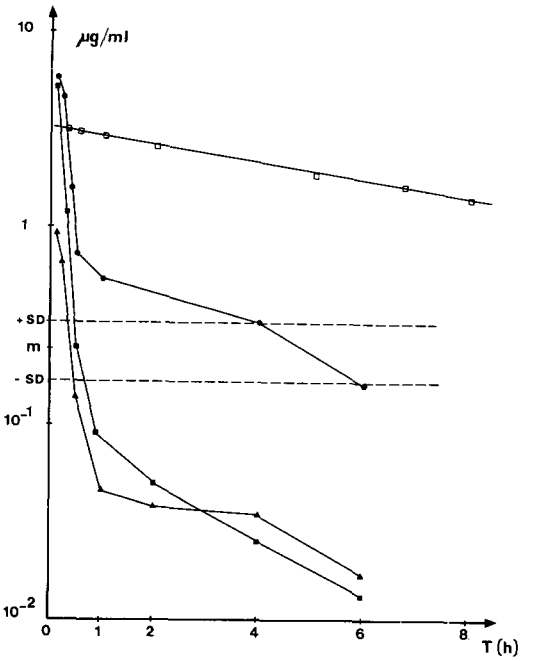

Fig. 1. Exogenous human (-■—■-), bovine (-▲—▲-), rat (-●—●-) Cu SOD and human Mn SOD (-□—□-) levels in rat plasma.

Exogenous SOD levels. Figure 1 shows the plasma levels of Cu SODs and Mn SOD administered i.v. as a function of time. After injection of 500 μg/kg of enzyme, the decay curves of human and bovine Cu SODs are similar and appear to be biexponential. Thirty minutes after injection, the heterologous immunoreactive Cu SOD level is less than 1% of initial level. The equation of plasma level $C(t)$, as a function of time, corresponding to the intravenous doses of 500 μg/kg can be written

$$\text{human Cu SOD: } C(t) = 10\ e^{(-0.1155t)} + 0.087\ e^{(-0057t)}$$

$$\text{bovine Cu SOD: } C(t) = 4\ e^{(-0.1155t)} + 0.056\ e^{(-0.0029t)}$$

After intravenous injection of an identical dose of rat Cu SOD, the initial half life of the decay curve is similar to those of heterologous enzymes and immunoreactive Cu SOD levels are not significantly different from basal endogenous values 30 min after the administration of the enzyme.

The blood pharmacokinetic characteristics of Mn SOD appear to be very different from those of exogenous Cu SODs, since the decay curve is monoexponential with a much longer period ($T_{1/2}$ = 6.45 hr).

Subcellular kidney cortex fixation of exogenous and endogenous Cu SODs. The subcellular fractionation was effected with kidney cortex excised 30 min after i.v. injection of iodinated bovine Cu SOD.

Table 1 shows the evolution of the radioactive concentration, expressed as c.p.m./mg of protein, in the course of the different steps of the differential

Table 1. Bovine I 125 I repartition in rat kidney cortex after i.v. injection of a tracer dose (3×10^6 c.p.m.)

	Proteins (mg)	c.p.m.	c.p.m./mg
Crude homogenate	223.1	763,478	3422
Filtered homogenate	131	473,648	3615
850 *g* supernatant	88.3	234,000	2650
9000 *g* pellet			
Bottom A	7.6	65,938	8676
Medium B	3.86	12,455	3226
Upper C	5.8	14,245	2458
9000 *g* Supernatant	50	87,408	1748

centrifugations of an homogenate of cortex. The radioactivity appears particularly located in the 9000 *g* pellet. The separation of this pellet in three fractions shows that the tracer is essentially located in the bottom layer. A similar result is obtained with human or murine tracer. The dilution of the iodinated Cu SOD in a pharmacological dose of non radioactive enzyme (500 μg/kg) does not modify the concentration of the radioactivity in the bottom layer (fraction A). Figures 2 (a)–(c) show the enzymatic and radioactive tracers obtained after isopycnic ultracentrifugation of fraction A. The separation between mitochondrial and lysosomes is shown by the different profiles of acid phosphatase and cytochrome oxidase (Fig. 2a). Use of a specific radioimmunoassay for rat Cu SOD shows that immunoreactive endogenous Cu SOD of the fraction A exhibits two peaks, one corresponding to that of cytochrome oxidase, the other one to that of acid phosphatase. Endogenous rat Cu SOD exhibits thus a double, mitochondrial and lysosomial, location in the kidney cortex.

The origin of lysosomial Cu SOD is evidenced after i.v. injection of rat 1 125 Cu SOD, since a single peak of radioactivity appears to correspond to that of acid phosphatase (Fig. 2b).

The intravenous administration of bovine Cu SOD (500 μg/kg) in the presence of a tracer dose of iodinated bovine Cu SOD leads to a similar result (Fig. 2c). As in the case of rat I 125 Cu SOD, the radioactivity is located in lysosomes. Moreover, the specific radioimmunoassay of bovine Cu SOD shows

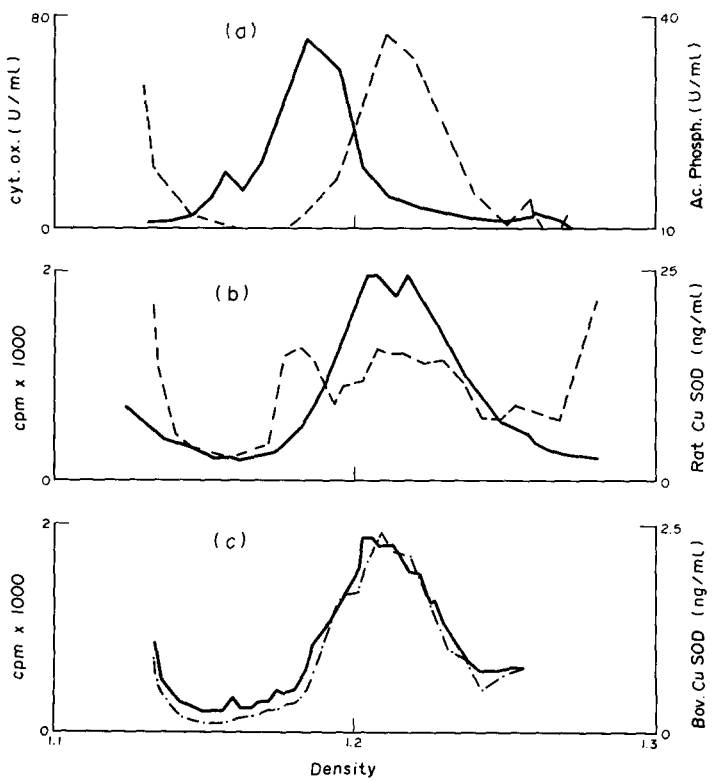

Fig. 2. Isopycnic centrifugation (30–60% saccharose) of 9000 *g* pellet: (a) acid phosphatase (·····) and cytochrome oxidase (——) activities; (b) endogenous (ng/ml) (·····) and exogenous (c.p.m.) rat Cu SOD (——); (c) exogenous immunoreactive (ng/ml) (·····) and radioactive (c.p.m.) bovine Cu SOD (——). Indicator enzymes were determined in the same runs as SOD distributions.

that the bovine enzyme is concentrated in the lysosomes.

Anti-inflammatory activity. Tables 2(A)–(D) show the results of *in vivo* anti-inflammatory activities of heterologous and isologous Cu SODs and human Mn SOD in the rat. For each series of experiments, the validity of the model was assessed by the highly significant activity of phenylbutazone (100 mg/kg), during both the prostaglandin and serotonin mediated phases. At the doses used (6.6, 33.3, 167 μg/kg) the anti-inflammatory activity highly depends upon the origin of the copper enzyme. Heterologous human and bovine Cu SODs exhibit a highly significant pharmacological activity (Tables 2A and 2B). However, the dose effect responses appear to be very different for both enzymes. Thus, purified human Cu SOD is efficient in both early and late phases for the highest doses (167 μg/kg), whereas the lowest doses show an anti-inflammatory effect during the early phase alone.

On the other hand, bovine Cu SOD is fully active at the lowest doses (33.3 and 6.6 μg/kg) and is inefficient at the highest dose (167 μg/kg).

The results obtained with isologous Cu SOD (Table 2C) are very different. Homologous rat Cu SOD i.p. administered is inefficient in both early and late phases and a transitory proinflammatory activity appears 1 hr after injection at 33.3 and 6.6 μg/kg.

The results obtained with human Mn SOD appear to be more complex. Indeed, although injection of 43.5 or 217 μg/kg shows no inhibitory action, the lowest dose (8.7 μg/kg) is able to suppress the early phase of oedema. On the other hand, a slight transitory proinflammatory activity appears 2 hr (217 μg/kg) after intraperitoneal injection (Table 2D).

DISCUSSION

The pharmacological properties and the mechanism of action of SODs must be interpreted as a function of their pharmacokinetic characteristics (bioavailability, tissue repartition, intracellular penetration, subcellular location). The supposed mechanism of anti-inflammatory activity relies on an increase of the extracellular concentration of this enzyme, thus leading to the scavenging of superoxide anion produced by activated phagocytes, and exerting both a deleterious cellular damage and an indirect chemotactic activity [3, 4]. In fact, the hypothesis according to which pharmacological activity would depend upon the biovailability of dismutase activity appears inconsistent with experimental data. Previous work [5] has shown a discrepancy between the evolution of injected bovine Cu SOD blood levels and the anti-inflammatory activity of the enzyme.

Moreover, the clinical effectiveness of SOD [18, 19] has been demonstrated for much lower doses of enzyme (100 μg/kg) than those described in experimental pharmacological studies [5, 16, 17].

The present results show that both copper and manganese containing dismutases exhibit contradictory pharmacokinetic and pharmacological properties. Indeed, injected human Cu SOD, shows an evident anti-inflammatory activity although the blood levels are low or even negligible when compared with rat plasma endogenous Cu SOD levels.

On the other hand, exogenous human Mn SOD

Table 2. Dose response in the carrageenan paw swelling in the rat of bovine (A) human (B) and rat (C) Cu SODs, and human Mn SOD (D), i.p. administered 30 min prior to injection of carrageenan

Time (hr)	Dose								
	6.6 μg.kg^{-1}			33.3 μg.kg^{-1}			166.6 μg.kg^{-1}		
	% Var.	t	P	% Var.	t	P	% Var.	t	P
(A)									
1	−79.2	6.0	0.001	86.7	6.4	0.001	−23.5	1.4	NS
2	−62.7	3.5	0.005	−51.5	3.3	0.005	−11.5	0.6	NS
3	−31.1	1.4	NS	−31.9	1.7	NS	−12.8	0.5	NS
5	−26.9	2.0	0.005	−62.5	5.5	0.001	− 4.5	0.3	NS
(B)									
1	−65.7	3.5	0.005	−75.5	4.1	0.001	−52.7	2.5	0.02
2	−35.0	1.8	0.1	−25.4	1.4	NS	−72.5	3.8	0.005
3	−18.1	1.1	NS	−20.2	1.2	NS	−55.8	3.3	0.005
5	−14.2	0.9	NS	−25.6	1.7	NS	−47.7	3.0	0.01
(C)									
1	+69.2	3.4	0.01	+70.0	3.3	0.01	+0.45	0.02	NS
2	+24.6	1.6	NS	+26.5	1.7	NS	+9.81	0.67	NS
3	+ 7.6	0.4	NS	+30.5	1.7	NS	+0.43	0.03	NS
5	+20.6	1.4	NS	+34.3	2.3	0.05	+3.00	0.23	NS
	8.7 μg.kg^{-1}			43.5 μg.kg^{-1}			217 μ.kg^{-1}		
(D)									
1	−73.71	3.75	0.01	−25.52	1.16	NS	−26.53	1.29	NS
2	−8.43	1.05	NS	+26.72	1.38	NS	+35.9	1.87	0.1
3	−6.04	0.32	NS	+34.62	1.99	0.1	+25.06	1.44	NS
5	−30.84	1.62	NS	+26.66	1.51	NS	+ 5.98	0.38	NS

Degrees of freedom = 28.

exhibits very different characteristics, since, due to its longer half life (6.45 hr) in the rat, higher dismutase levels than those due to endogenous Cu SOD are obtained after i.v. administration. In spite of these properties, Mn SOD does not exhibit any significant anti-inflammatory activity.

These data could be interpreted by differences between pharmacological and/or pharmacokinetic properties of heterologous and homologous exogenous Cu SODs. The present results are consistent with this hypothesis since they show a discrepancy between pharmacological properties of fully active heterologous enzymes and inefficient isologous enzymes in the rat.

Thus, the dose response effects of heterologous Cu SODs depend upon the origin of the protein, since bovine enzyme is efficient at doses 5 times lower than human enzyme. On the other hand, for identical doses, homologous exogenous Cu SOD appears devoid of significant anti-inflammatory activity.

These data cannot be explained (1) by differences in specific enzymatic activities, since their values determined according to [10] are in a close range (2900–3200 riboflavin NBT U/mg) for bovine, human and rat enzymes, (2) by differences of pharmacokinetic properties. The decay curves of plasma exogenous Cu SODs are similar for the three enzymes, and the mechanisms of kidney fixation appear to be similar. After i.v. administration of tracer or pharmacological doses, the enzymes are filtered, reabsorbed in proximal tube and then rapidly internalized in lysosomes, as shown by the analysis of saccharose gradients of kidney cortex homogenates. It remains possible that the lysosomial metabolization leads to metabolites with different biological activities according to the origin of the protein.

It appears that the level of circulating exogenous SOD is not at all correlated with anti-inflammatory activity. Differences in pI values of the different enzymes cannot be invoked as an exclusive explanation and it may be that the major factor is fixation of very few molecules of exogenous enzyme to cell membranes. In this respect it has been demonstrated that protection of human fibroblasts against u.v. damage by bovine Cu SOD is unchanged even when excess exogenous SOD is removed by washing [20].

The present results thus show that the anti-inflammatory activity of exogenous Cu SOD is indirect and unrelated to the appearance and the maintaining of a high extracellular dismutase activity levels. This problem is complicated by the recent demonstration of an extracellular Cu SOD (M.W.135,000) which is present particularly in rat serum [21].

The pharmacological properties of exogenous Cu SODs appear thus limited in the rat to heterologous enzymes. This result may have important consequences, since clinical assays in man were effected with bovine enzyme. Although all studies concord on the safety of these treatments, the risk of immunological complications must not be ruled out [22]. Treatment with human enzyme would avoid this problem, but the pharmacological activity of human Cu SOD in man remains to be established, particularly if a certain distance in terms of sequence homology between endogenous and exogenous enzymes is necessary. It is to be noted that the present work describes results with a single animal inflammation model, using levels of SOD corresponding to human clinical doses (about 60–100 μg/kg), rather than the very much higher levels used by other workers. However, preliminary results show that the pharmacological activity of similar doses of Cu SOD is also restricted to heterologous enzymes with Freund adjuvant-induced arthritis model in the rat.

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