Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species

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The contents of extracellular superoxide dismutase, CuZn superoxide dismutase and Mn superoxide dismutase were determined in tissues from nine mammalian species. The pattern of CuZn superoxide dismutase distribution was similar in all species, with high activity in metabolically active organs such as liver and kidney and low activity in, for example, skeletal muscle. Mn superoxide dismutase activity was high in organs with high respiration, such as liver, kidney and myocardium. Overall the Mn superoxide dismutase activity in organs was almost as high as the CuZn superoxide dismutase activity. The content of extracellular superoxide dismutase was, almost without exception, lower than the content of the other isoenzymes. The pattern of tissue distribution was distinctly different from those of CuZn superoxide dismutase and Mn superoxide dismutase. The tissue distribution of extracellular superoxide dismutase differed among species, but in general there was much in lungs and kidneys and little in skeletal muscle. In man, pig, sheep, cow, rabbit and mouse the overall tissue extracellular superoxide dismutase activities were similar to each other, whereas dog, cat and rat tissues contained distinctly less. There was no general correlation between the tissue extracellular superoxide dismutase activity of any of the various species and the variable plasma activity. The ratio between the plasma and the overall tissue activities was high, for some species over unity, providing further evidence for the notion that one role of extracellular superoxide dismutase is as a plasma protein.

Recently a high- M_r factor with SOD (EC 1.15.1.1) activity was demonstrated in plasma from several mammalian species (Marklund *et al.*, 1982b). A similar factor was subsequently isolated from human lungs (Marklund, 1982) and named EC-SOD after the location where it was first detected, i.e. extracellular fluids. The plasma and lung factors behaved apparently identically in the six protein separation steps that have been tested and are probably identical. EC-SOD is a tetrameric, slightly hydrophobic, glycoprotein with an M_r of 135000. On chromatography on heparin-Sepharose it is separated into three fractions, A, B and C, with different affinities for heparin. The enzyme has a high specific activity, is cyanide-sensitive, contains four copper atoms/molecule

and probably also four zinc atoms/molecule (Marklund, 1982). No correlation with other SOD isoenzymes has been found with respect to amino acid composition, antigenic properties (Marklund, 1984) and probably also chromosomal localization (Marklund *et al.*, 1982b).

In plasma, EC-SOD is by far the dominant SOD isoenzyme in most mammalian species examined. There were very large differences in plasma content among the species (Marklund *et al.*, 1982b). For the unravelling of the roles of EC-SOD, it was judged important to make a comprehensive analysis of the occurrence of EC-SOD in tissues from a number of species. The present paper reports on the EC-SOD content in tissues from nine mammalian species. For comparison, the contents of the previously known mammalian SOD isoenzymes, CuZn-SOD (McCord & Fridovich, 1969) and Mn-SOD (McCord *et al.*, 1977; Marklund, 1978), were also determined.

Abbreviations used: SOD, superoxide dismutase; EC-SOD, extracellular superoxide dismutase.

Materials and methods

Materials

Concanavalin A-Sepharose, CNBr-activated Sepharose 4B and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). KO_2 was a Ventron (Karlsruhe, Germany) product, and α -methyl D-mannoside was obtained from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). All other chemicals were of analytical reagent grade. Water was double-distilled from glass vessels.

Tissues

Human tissues were obtained within 24 h after death from accident victims without known disease at the Department of Forensic Medicine, Umeå University Hospital. Tissues from pig, sheep and cow were obtained fresh from the local abattoir. Cat, dog and rabbit tissues were taken from animals specially bred for laboratory purposes. The rats were of the R strain and the mice were of the BALB strain. All tissues were kept at -80°C until preparation.

Extraction of EC-SOD

Several procedures for extraction were tested. Sodium acetate buffer (50 mM), pH 5.5, extracted about as much EC-SOD but less total protein from human lungs than the routine SOD extraction buffer used in the laboratory, namely 10 mM-potassium phosphate buffer, pH 7.4, containing 30 mM-KCl. Triton X-100 (0.3%) in the acetate buffer decreased the yield of EC-SOD activity. In view of the heparin affinity of EC-SOD (Marklund, 1982), heparin and dextran sulphate (0.4 mg/ml) in the acetate buffer were tested but were found to be without effect. Addition of the chaotropic salt KBr to the acetate buffer increased the yield of EC-SOD up to 2–3-fold. At concentrations of KBr above 0.25 M no further increase in extraction was noted. Extraction of CuZn-SOD and Mn-SOD was as effective with 50 mM-sodium acetate buffer, pH 5.5, as with 10 mM-potassium phosphate buffer, pH 7.4, containing 30 mM-KCl, and was not influenced by KBr. On the basis of the above investigation, the following procedure was adopted. The tissues were homogenized in 10 vol. of sodium acetate buffer, pH 5.5, containing 0.3 M-KBr, in an Ultra-Turrax homogenizer. The homogenates were then sonicated and finally extracted for 30 min at 4°C . The supernatants after centrifugation (20 000 g for 15 min) were employed for the further analyses.

Separation of SOD isoenzymes by concanavalin A-Sepharose chromatography

Unlike CuZn-SOD and Mn-SOD, EC-SOD binds to concanavalin A, probably because of the

presence of carbohydrate. The samples (1–2 ml) were applied to a concanavalin A-Sepharose column (1 cm $\times$ 1 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 6.5, containing 120 mM-NaCl. The samples were divided into 0.5 ml portions and applied at 5 min intervals to allow binding to the lectin. After 5 min, 2 ml of the phosphate buffer containing NaCl was added. The eluate from the homogenate and buffer additions was collected and contained the CuZn-SOD and Mn-SOD of the sample. After that, the column was washed with 20 ml of the phosphate buffer containing NaCl. The EC-SOD was then eluted with 5 ml of 150 mM- α -methyl D-mannoside in 50 mM-sodium phosphate buffer, pH 6.5, added in 1 ml portions at 5 min intervals. The column was regenerated with 5 ml of 0.5 M- α -methyl D-mannoside followed by 20 ml of 10 mM-potassium phosphate buffer, pH 6.5, containing 120 mM-NaCl. The yield of EC-SOD from the column, tested with pure EC-SOD as well as with partially purified enzyme, was regularly about 75%. All values presented are compensated for this. Repeated separations of tissue extracts indicated a relative standard deviation in the determination of EC-SOD activity of 9%.

Separation of SOD isoenzymes by gel chromatography

The chromatography was performed in a 1.6 cm $\times$ 90 cm column of Sephacryl S-300 at 6 ml/h with 10 mM-potassium phosphate buffer, pH 7.4, containing 150 mM-NaCl as eluent. The tissues were extracted as described above and dialysed against the elution buffer overnight. About 5 ml of tissue extract was applied.

SOD analysis

SOD was determined in terms of its ability to catalyse the disproportionation of $\text{O}_2^{\cdot-}$ in alkaline aqueous solution. The disproportionation was directly studied in a spectrophotometer, essentially as described previously (Marklund, 1976), except that all isoenzymes were assayed at pH 9.50 and that 3 mM-cyanide was used to distinguish between the resistant Mn-SOD and the sensitive isoenzymes CuZn-SOD and EC-SOD. One unit in the assay is defined as the activity that brings about a decay in $\text{O}_2^{\cdot-}$ concentration at a rate of 0.1 s^{-1} in 3 ml of buffer. It corresponds to 8.3 ng of human CuZn-SOD, 8.8 ng of human EC-SOD and 65 ng of bovine Mn-SOD. The xanthine oxidase/cytochrome c assay for SOD activity works at physiological conditions, i.e. neutral pH and low $\text{O}_2^{\cdot-}$ concentration (McCord & Fridovich, 1969). When bovine and human enzymes are analysed, 1 unit in the present assay method corresponds to 0.024 units of CuZn-SOD and EC-SOD and 0.24

units of Mn-SOD in the 'xanthine oxidase' assay method. The present assay method is thus about 10 times more sensitive for CuZn-SOD and EC-SOD activity than for Mn-SOD activity.

Results

SOD isoenzymes in tissue homogenates

Table 1 collects the results of determination of the SOD isoenzymes in tissues from nine mammalian species. The data are based on the separation of the tissue homogenates on concanavalin A-Sepharose as described in the Materials and methods section. As shown, EC-SOD activity was demonstrated in all investigated tissues in all species. Except for mouse lung, the EC-SOD content was lower than the contents of the other SOD isoenzymes.

EC-SOD estimated by gel chromatography

The separation of EC-SOD indicated in Table 1 was based on knowledge of the concanavalin A affinity of human EC-SOD (Marklund, 1982). EC-SOD from other species has not been isolated and characterized. To validate the data it was judged important to use another property of the EC-SOD molecule to achieve separation from the other

isoenzymes. EC-SOD has a higher M_r than the other isoenzymes, and a number of the tissue homogenates were therefore subjected to gel chromatography. Fig. 1 shows as an example gel chromatography of a cow lung homogenate. The small high- M_r peak has a position corresponding to the M_r of EC-SOD. The peak accounts for about 5.5% of the total SOD activity of the chromatogram. This is in good agreement with the estimation of EC-SOD by means of concanavalin A-Sepharose, which gave 5.0%. The results of comparison between gel chromatography and concanavalin A-Sepharose analysis of ten other tissue homogenates are shown in Table 2. When judging the data it must be realized that precise determination of EC-SOD by means of gel chromatography is difficult, especially when the content is low relative to CuZn-SOD. On the whole, the agreement between the two procedures is good, which indicates that the concanavalin A-Sepharose procedure is valid.

Plasma SOD activity

The SOD activities of cow and sheep plasma are presented in Table 3. The SOD activities of plasma from the other species encompassed in the present investigation have been presented before (Marklund *et al.*, 1982b), but are included in the Table to facilitate evaluation and discussion of the data.

Fig. 1. Gel chromatography of a calf lung homogenate

A calf lung homogenate was chromatographed on Sephacryl S-300 as described in the Materials and methods section. ○, A_{280} ; ▲, SOD activity.

Table 1. *Content of SOD isoenzymes in tissues from nine mammalian species*

The isoenzymes were separated by the concanavalin A-Sepharose procedure and analysed as described in the Materials and methods section. The activity was analysed on tissues from two individuals of each species, except for the mouse, for which to obtain enough material for handling and separation on concanavalin A-Sepharose tissues from three individuals were pooled and two different pools analysed. It should be noted that the sensitivity of the SOD assay method is about 10 times lower for Mn-SOD activity compared with CuZn-SOD and EC-SOD activity. On the lowest row the mean activities $\pm$ s.d. for all the tissues are presented. The values were calculated from the mean activity (of the two individuals) of each tissue.

SOD activity (units/g wet wt.)															
	Man			Pig			Sheep			Cow			Dog		
	EC-SOD	CuZn-SOD	Mn-SOD	EC-SOD	CuZn-SOD	Mn-SOD	EC-SOD	CuZn-SOD	Mn-SOD	EC-SOD	CuZn-SOD	Mn-SOD	EC-SOD	CuZn-SOD	Mn-SOD
Gut	570	10000	358	249	8170	218	—	—	—	350	9850	233	29	8600	240
	220	7300	256	92	15100	186	—	—	—	460	9440	270	4	9180	295
Heart	251	11300	1250	90	6740	1290	64	6630	2210	54	6850	2330	24	6810	2070
	196	15800	1480	87	6000	811	90	9310	2190	175	6290	1160	8	9270	2280
Kidney	282	24800	1510	1090	41500	2130	166	41300	3070	248	21300	1800	17	24400	3100
	345	31320	1660	518	29100	1110	301	38000	3110	519	37400	1950	9	35400	3920
Liver	80	106900	2260	322	74500	1540	284	119000	4670	144	75200	1850	74	61100	2970
	57	63000	2300	155	72600	1110	248	200000	2550	481	114000	1540	8	52300	4010
Lung	793	7500	331	221	6480	160	779	31800	111	357	11800	279	68	8000	174
	301	7700	410	340	8700	270	952	32800	284	640	19400	119	6	8100	263
Lymphatic gland	763	7750	653				186	10400	339	361	7130	236	22	35500	1760
	184	8680	644	119	12200	146	189	11000	453	275	11400	349	4	7100	460
Pancreas	619	8630	778	154	8200	667				271	13300	964	5	4100	480
	684	11300	905	468	10000	618				270	11700	850	2	9720	755
Skeletal muscle	96	12900	552	27	2970	376	99	5100	725	35	5710	836	30	5500	1190
	53	12700	374	43	2180	141	188	6410	931	186	8000	460	2	7700	840
Spleen	68	13300	285	162	13500	211				280	11400	84	9	9800	197
	106	13200	384	207	8900	182				301	13300	83	4	11700	461
Thymus				30	11600	133	48	5150	89	30	8820	202			
				47	12700	72	84	9560	184	115	14600	490	6	7500	451
Thyroid gland	1480	10700	276	219	21500	140				31	8950	231	16	10400	525
	915	14500	276										5	10400	291
Mean	403	19500	847	226	18500	536	258	37600	1490	278	20800	804	17	15900	1240
	346	23400	680	212	20300	546	278	55400	1455	139	26700	709	12	15400	1250

SOD activity (units/g wet wt.)												
	Cat			Rabbit			Rat			Mouse		
	EC-SOD	CuZn-SOD	Mn-SOD	EC-SOD	CuZn-SOD	Mn-SOD	EC-SOD	CuZn-SOD	Mn-SOD	EC-SOD	CuZn-SOD	Mn-SOD
Gut	100	6760	525	234	25700	0	20	11700	268	140	22000	14
	82	9090	339	182	12200	429	17	6840	199	113	18900	58
Heart	84	17100	2940	231	10300	2220	34	19600	2830	196	15700	2490
	53	13100	2940	182	9600	2290	36	10600	1420	190	13600	2410
Kidney	29	35900	3270	551	39100	2790	51	49900	2090	364	33600	1300
	22	30500	2210	393	42800	2680	108	52700	1070	468	30700	1660
Liver	60	44700	4370	274	65000	1210	19	95300	1800	321	91700	1380
	60	56800	4090	375	56400	2105	20	96000	2300	280	70200	1310
Lung	48	9850	487	227	14100	160	113	8160	165	3320	30700	307
	91	8030	406	213	15200	191	121	8130	135	3130	18400	173
Lymphatic gland												
Pancreas	45	8590	1150	417	18000	719	46	12300	909	260	16100	316
	35	10700	723	193	5650	196	55	13100	1240	240	14900	333
Skeletal muscle	63	4240	332	117	4000	37	33	3530	105	44	7310	269
	30	7440	165	105	7100	162	30	4690	137	65	6840	144
Spleen	36	13300	447	339	18000	363	20	18200	247	93	18900	98
	31	13200	279	308	20400	303	11	13300	177	126	20100	99
Thymus				350	4470	54	48	8750	77	348	10900	0
	5	12700	79	233	11100	114	55	7640	119	533	9960	146
Thyroid gland				412	7600	86						
				948	5300	80						
Mean	49	17500	1450	314	19600	809	47	24500	854	563	25000	695
	26	14800	1590	161	17700	1010	33	30200	876	988	22300	858

Table 2. Comparison of EC-SOD determined by concanavalin A-Sepharose chromatography and by gel chromatography. EC-SOD was determined by the two procedures as described in the Materials and methods section. The results are presented as percentages of total SOD activity of the tissue homogenates.

	EC-SOD determined by concanavalin A-Sepharose chromatography (%)	EC-SOD determined by gel chromatography (%)
Human heart	1.9	0.6
Human lung	8.8	6.5
Pig lung	6.6	8.1
Pig kidney	2.1	3.1
Sheep lung	2.4	2.4
Cow lung	5.0	5.5
Dog lung	0.9	1.8
Cat lung	0.5	0.7
Rabbit lung	1.4	0.7
Rat lung	0.9	*
Mouse lung	9.9	10.5

* No distinct peak discernible, but extensive streaking of the large CuZn-SOD peak.

Table 3. SOD activity of plasma from nine mammalian species

The plasmas were analysed with the KO_2 assay. The values given here for EC-SOD are the cyanide-sensitive activities of the plasma specimens. Plasma samples from all species were separated on Sephacryl S-300, and practically all activity was given by the high- M_r peak. The activity at the position of CuZn-SOD (M_r about 30000) was negligible.

	No. of animals	EC-SOD (units/ml)	Activity in 3mM-cyanide (Mn-SOD) (units/ml)	CuZn-SOD (units/ml)
Rabbit*	4	636 ± 207	10.0 ± 4.4	low
Mouse*	4	400 ± 60	7.1 ± 2.1	low
Rat*	5	332 ± 23	7.1 ± 2.1	low
Cow	4	126 ± 14	5.7 ± 1.6	low
Sheep	6	97.9 ± 5.8	8.8 ± 0.8	low
Pig*	5	56.0 ± 14	6.5 ± 0.8	low
Man*	51	26.3 ± 3.6	3.4 ± 0.5	$1.3 \pm 0.7^\dagger$
Dog*	5	8.7 ± 3.4	4.5 ± 1.1	low
Cat*	5	4.1 ± 2.4	5.5 ± 1.9	low

* Values for these species taken from Marklund *et al.* (1982b).

† The content was analysed by a radioimmunoassay method (Marklund *et al.*, 1982b) and the value converted into units by using the specific activity of pure human CuZn-SOD.

Discussion

The results in Table 1 represent the most comprehensive analysis presented so far of the CuZn-SOD, Mn-SOD and EC-SOD contents in various species. The data might be useful for the interpretation of the results, when the sensitivities of different tissues and different species to potentially oxy-radical-producing systems are compared. For example, the higher resistance of rabbit lungs compared with rat and human lungs to paraquat and hyperbaric oxygen (Martin *et al.*, 1981) correlates with a higher CuZn-SOD content shown in Table 1.

Some general patterns in terms of tissue isoenzyme distribution are seen. The CuZn-SOD activity is very high in liver from all species, with the

likewise metabolically active kidney as a strong second. The contents in skeletal muscle are in general low, whereas the other tissues display similar intermediate activities. The Mn-SOD contents of liver, kidney and heart are similar and very high, whereas thymus, gut, thyroid gland and spleen are located on the other end of the scale. There is no general correlation between the tissue contents of CuZn-SOD and Mn-SOD. For example, there are high CuZn-SOD and Mn-SOD contents in liver and kidney, but that correlation does not hold for heart. Pancreas contains comparatively high Mn-SOD contents but not particularly much CuZn-SOD.

When the different species are compared, some patterns emerge. Sheep tissues contain very much CuZn-SOD. Mouse and rat tissues are also

abundant in CuZn-SOD. On the other end of the scale we find dog, cat and pig tissues. Cat, sheep and dog tissues, in general, contain much Mn-SOD whereas pig, mouse and cow tissues contain little. There is no general positive or negative correlation between the isoenzymes; sheep tissues contain much of both, whereas pig tissues contain little of both. The previously described large species differences in liver Mn-SOD, with much in human liver and negligible amounts in rat and bovine liver (McCord *et al.*, 1977), were not observed here. The sensitivity of the SOD assay is about 10-fold lower for Mn-SOD than for CuZn-SOD and EC-SOD. When that fact is allowed for, it is evident that Mn-SOD in general accounts for a substantial part of the total SOD activity of tissue homogenates.

As a whole, there is much less EC-SOD in the tissues than there is of the other isoenzymes. The only significant exception to that rule is the high EC-SOD content in mouse lung, which exceeds that of Mn-SOD. For most of the species investigated, namely man, pig, sheep, cow, rabbit and mouse, the EC-SOD contents overall are similar to each other, and account for in average 1–1.5% of the cyanide-sensitive SOD activity of the tissues. The highest individual value observed is the 11.5% for mouse lungs. Dog, cat and rat tissues contain distinctly less. Whether the tissue contents really are so much lower, or depend on problems with the extraction and detection, cannot be judged with certainty. The effect of different KBr concentrations (0–0.9M) on the extraction of EC-SOD from dog and rat lungs was tested, but no improvements over the basal procedure were noted.

If the tissues from the different species are compared, some similarities are found. Lungs and kidneys, in general, contain much, whereas skeletal muscles are poor in, EC-SOD. On the other hand, highly variable amounts are detected, for example, in liver and pancreas. These are species differences, since the investigated individuals within each of the various species are similar. On the whole, the tissue distribution of EC-SOD differs more between species than do the distributions of CuZn-SOD and Mn-SOD. The EC-SOD distribution has a pattern distinct from those of the other SOD isoenzymes, and shows, for example, no correlation with the metabolic activity of the tissues.

As to the role(s) of EC-SOD in the body, no definite information is obtained from the present investigation. However, the data strengthen the idea that one role is as a plasma protein and hence as a protector in the extracellular space. It has previously been shown that EC-SOD is the major SOD isoenzyme in extracellular fluids, and it is a glycoprotein, like most plasma proteins. Although there is a large interspecies variation, the variation

in plasma activity among individuals within any one of the various species is small, giving the impression that the concentration is regulated (Table 3). It is curious that there is no definite correlation between the tissue contents and the plasma contents (Tables 1 and 3). Rabbits and mice have much both in plasma and tissues, man has little in plasma and much in tissues, rats have much in plasma and little in tissues, and dogs and cats have little both in plasma and in tissues. On the other hand, the ratios between the tissue contents as units/g wet wt. and plasma contents as units/ml are distinctly different from those for the other SOD isoenzymes. In man the ratio for CuZn-SOD is about 15000 (Tables 1 and 3) and for Mn-SOD several hundred (Tables 1 and 2; Baret *et al.*, 1981; Nishimura *et al.*, 1982). The presence of these isoenzymes in plasma is in all probability the result of passive leakage from cells, and the ratios are similar to those seen for other intracellular enzymes. In man the ratio for EC-SOD is much smaller, about 15, and for some other species listed in Table 3 it is near unity and even lower. These facts are strong evidence for the notion that one intended role of EC-SOD is as an extracellular protein. As to the location of tissue EC-SOD, little can be deduced from the present data. The slight hydrophobicity (Marklund, 1982) and the fact that the extraction from tissues is improved by a chaotropic salt might indicate that at least part of tissue EC-SOD is associated with cellular membranes.

In the extracellular space there are many potential sources of $O_2^{\cdot-}$. All leucocytes, except lymphocytes, produce $O_2^{\cdot-}$ on activation (Halliwell, 1982). There is evidence for $O_2^{\cdot-}$ -producing clastogenic factors in plasma in autoimmune and other types of disease (Emerit, 1982). At re-perfusion after a period of ischaemia there appears to be a burst of oxy-radical production (Parks *et al.*, 1982; Gardner *et al.*, 1983). In contrast, there appears to be little enzymic protection against oxy radicals in the extracellular space. The catalase activity is negligible (Marklund *et al.*, 1982a), and there is no functional glutathione peroxidase activity. Caeruloplasmin may be of importance as a protector against oxygen toxicity (Gutteridge & Stocks, 1981). The SOD activity is very low. The extracellular space may be an especially likely site in the body for oxy-radical-mediated damage. It is possible that the low but apparently regulated SOD activity should be seen as a compromise between the need to protect the extracellular-fluid components and cell surfaces against $O_2^{\cdot-}$ and the importance of not interfering unduly with beneficial and intended effects of oxy radicals. The latter may include microbicidal and cytotoxic activity of leucocytes (Johnston *et al.*,

1975; Murray *et al.*, 1979; Halliwell, 1982), activation of natural killer cells (Helfand *et al.*, 1983) and chemotactic activity induced by $O_2^{\cdot-}$ (Petrone *et al.*, 1980). For some reason, then, that compromise has been reached at different plasma EC-SOD activities in different species.

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