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Changes in Superoxide Dismutase Activity in Liver and Lung of Old Rats

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Superoxide dismutase activity was measured in liver and lung from 3 and 24 month-old rats. Both total SOD and Mn-SOD activity decreased significantly in the liver of old rats. Recent results from our laboratory have indicated that during aging, the activity of Cu/Zn-SOD decreases in rat liver and that there is an accumulation of altered protein. It was also shown that the old Cu/Zn-SOD had one histidine fewer than the young one. In the present study, the immunoprecipitation experiments showed that the amount of immunoprecipitable Mn-SOD from liver of old rats was greater than from young ones, but when amino acid residues were measured in purified young and old Mn-SOD from liver, no change was observed. In lung, no significant age-related differences in total SOD, Cu/Zn-SOD and Mn-SOD activity were found, nor was there accumulation of altered protein during aging.

Keywords: Superoxide dismutase, aging, antioxidant defense, oxidative damage

Abbreviations: NBT: Nitro Blue Tetrazolium; PMSF: Phenyl Methane Sulfonyl Fluoride; SOD: Superoxide Dismutase

INTRODUCTION

Oxidative damage has been implicated as one of the principal causes of age-related oxidative inactivation of enzymes.[1] Such damage is at least partially associated with the free radical, superoxide $O_2^{\cdot}$,[2] which seems to be increased in aging.[3] The primary defense against the toxic effects of the superoxide radical is its dismutation by superoxide dismutase (SOD).[2] Three metalloproteins have been found to exhibit SOD activity. The Cu/Zn-containing enzyme (Cu/Zn-SOD) is found in all eukaryotes, land plants and fungi, while a Mn-containing SOD (Mn-SOD) has been found in bacteria as well as in the mitochondrial matrix of plants and animals.[4] The third enzyme, a Fe-containing SOD (Fe-SOD), is found predominantly in prokaryotes.[4]

Alterations in a large number of enzymes are found in tissues of old animals.[5–9] Many of these

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changes result in the accumulation of the inactive or less active forms of the enzymes.[7–9] In a previous report[9] we demonstrated that during aging, the activity of Cu/Zn-SOD decreases in rat liver and there is an accumulation of inactive or less active forms. That study also demonstrated that the old enzyme has one histidine fewer than the young one. Thus, the decrease of specific activity of Cu/Zn-SOD during aging could be due to chemical modification of the histidine residues, probably caused by oxidation.

In the present paper, we extend this study to the Mn-SOD in liver. In addition we study the behavior with age of Cu/Zn and Mn-SOD in the lung, an organ which is exposed to a greater oxidative stress.[10] Our results show an age-related decrease in Mn-SOD activity in liver and no age-related differences in SOD activity in lung. The increase in the amount of inactive form seems not to be associated to the loss of the most oxidation-susceptible amino acid residues -histidine and lysine-[1,7,8] studied.

MATERIALS AND METHODS

Animals

Young (3 months) and old (24 months) male Wistar rats were used. They were maintained on a standard laboratory diet with free access to food and water.

Enzyme Assay

Cu/Zn-SOD and Mn-SOD were measured using the xanthine-oxidase-cytochrome c method as described by McCord & Fridovich.[11] The final concentrations in the cuvettes were 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 10 μM cytochrome c, 50 μM or 2 mM cyanide, 1 U catalase and tissue sample. The reaction was initiated by the addition of 1 U xanthine-oxidase. The inhibition of cytochrome c reduction was followed spectrophotometrically at 550 nm. One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of cytochrome c reduction by 50%. The activities are expressed as units/mg of protein. Inhibition of Cu/Zn-SOD by cyanide allows for the differential quantification of both Cu/Zn-SOD and Mn-SOD. Protein concentrations were determined by the method of Lowry *et al.*[12]

Enzyme Purification

Cu/Zn-SOD and Mn-SOD from liver were purified according to the method described by Kohtaro and Burr[13] and Salin *et al.*[14] Electrophoresis analysis was carried out by the procedure of Laemmli.[15] Cu/Zn-SOD was assayed in acrylamide gel according to the method previously described by Nishikimi *et al.*[16] and Mn-SOD activity by the method previously described by Beauchamp and Fridovich.[17]

Purification of Cu/Zn-SOD and Mn-SOD from Young and Old Rats by Immunoaffinity-chromatography

The homogenate was dialyzed in 5 mM potassium phosphate buffer, pH 7.2, 0.1 mM EDTA and 5 μM PMSF. The dialyzed supernatant was applied to an immunoaffinity column (1.5 × 6 cm, Sepharose 4B CNBr activate, Pharmacia LKB Biotechnology Inc.) prepared with antibodies against Cu/Zn-SOD and Mn-SOD respectively, equilibrated with 5 mM potassium phosphate buffer (pH 7.2), 0.1 mM EDTA and 5 μM PMSF. The enzyme was eluted from the column with a 5–50 mM linear gradient of potassium phosphate buffer.

Antibody Iodination and Immunoprecipitation

The antibody was radioactively labeled with ^{125}I by the chloramine-T method according to Greenwood *et al.*[18] Immunoprecipitation experiments were carried out by incubating 100 mU of SOD with increasing volumes of iodinated antibody (0 to 100 μl). The mixture was adjusted to a constant volume with 1.7% (v/v) Triton X-100 and 150 mM NaCl.

The mixtures were allowed to react for 1 h at 37°C and overnight at 4°C. The reaction mixture was then centrifuged at 12,000 × g for 10 min and the remaining SOD activity was assayed in the supernatant. The precipitates were washed twice with 150 mM NaCl. The radioactivity incorporated into the precipitate was counted in a Gamma cord II.

Chemical Modification

Sulphydryl groups were determined by the method of Ellman.[19] Arginine residues were determined by the method described by Yamasaki *et al.*[20] Histidine residues were quantified using the diethylpyrocarbonate method described by Tophan and Dalziel.[21] Lysine residues were determined by the method of Tuengler and Pfletderer.[22]

RESULTS

Age-dependent Changes in SOD Specific Activities in Liver and Lung

SOD activities were measured in homogenates of liver and lung from young (3 months) and old (24 months) rats. In old rats a significant decrease in total SOD in liver (16%) was observed, whereas no significant age-related difference in total SOD was found in lung. We previously demonstrated that during aging, the activity of Cu/Zn-SOD decreases in rat liver.[9] However, no significant age-related difference in Cu/Zn-SOD activity was found in lung (Table 1). On the other hand, in old rats the Mn-SOD specific activity decreased 16% in liver, whereas no significant age-related difference in Mn-SOD was found in lung (Table 1).

Age-dependent Changes in Amount of Cu/Zn-SOD and Mn-SOD

In lung, the amount of Cu/Zn-SOD at both ages was studied by immunotitration. The polyclonal antibodies used were prepared against purified young rat Cu/Zn-SOD. Increasing amounts of the antiserum were added to the same amount of Cu/Zn-SOD units. No significant differences were observed in this tissue, so, there is no accumulation of a less active form of Cu/Zn- SOD during aging in lung (Table 1). In liver, we have recently reported an accumulation of inactive or less active form.[9]

The amount of Mn-SOD at both ages was also studied by immunotitration in liver and lung. The amount of antiserum needed to inactivate 100% of the activity was higher in older animals in liver (120%), whereas no significant difference was observed in lung (Table 1).

TABLE I Specific Activities and Immunoprecipitable Amount of SOD in Liver and Lung from Young and Old Rats.

	Young Rats	Old Rats
Specific activity		
Liver Total SOD	31.02 ± 0.45	26.18 ± 0.70**
Liver Mn-SOD	16.67 ± 0.51	13.95 ± 0.49**
Lung Total SOD	5.92 ± 0.81	5.23 ± 1.00
Lung Cu/Zn-SOD	3.42 ± 1.22	3.10 ± 0.57
Lung Mn-SOD	2.55 ± 1.08	2.21 ± 1.08
Amount of enzyme		
Liver Mn-SOD	3,622 ± 141	4,366 ± 163**
Lung Cu/Zn-SOD	2,102 ± 356	1,924 ± 184
Lung Mn-SOD	1,650 ± 690	1,723 ± 399

Results of specific activities are expressed as U/mg protein as mean ± SEM of five determination. Immunoprecipitable protein is expressed as cpm as described in Materials and Methods. Statistical significance: Student's test. **$p < 0.001$.

Study of Amino Acid Residues of Mn-SOD in Liver from Young and Old Rats

In order to investigate the possible mechanism underlying the loss of Mn-SOD specific activity during aging, in liver, various amino acid residues were quantified in Mn-SOD preparations purified by immunoaffinity chromatography from young and old rat (Table 2). The results show that there are no age-dependent changes in the amino acid residues studied. A previous report showed that the old Cu/Zn-SOD from liver has one histidine fewer than the young one, probably as a result of oxidation.[9]

TABLE II Amino Acid Residues per mol of Liver Mn-SOD Purified from Young and Old Rats.

Residue	State of Protein	Young Rats	Old Rats
-SH	Native	2.21 ± 0.23	1.90 ± 0.39
	Denatured	3.06 ± 0.38	2.38 ± 0.27
Arginine	Native	2.76 ± 0.30	2.83 ± 0.27
	Denatured	4.77 ± 0.16	4.48 ± 0.35
Histidine	Native	5.21 ± 0.26	4.79 ± 0.27
	Denatured	8.11 ± 0.27	8.47 ± 0.40
Lysine	Denatured	14.17 ± 2.03	16.35 ± 1.91

Mn-SOD was denatured with 6 M guanidinium chloride. Results are mean ± SEM of five determinations.

DISCUSSION

The antioxidant defense mechanism seems to be decreased in the elderly.[23] However, the literature is controversial with respect to the activity of SOD during aging. Thus, SOD total, Cu/Zn-SOD and Mn-SOD have been reported to either increase, remain unchanged, or decrease in aging (for review see ref. 24).

Recently we have reported that the activity of Cu/Zn-SOD decreases in rat liver with age. An accumulation of inactive or less active forms was shown; besides, we found that the enzyme from aged animals had a histidine fewer than one from young animals.[9] In the present paper we extend this study to the Mn-SOD enzyme. An age-dependent decrease in the specific activity of Mn-SOD from rat liver was observed (Table 1). Other authors have also reported a decrease in liver Mn-SOD of male rats in the elderly.[25,26] In contrast Rikans *et al.*[27] found an increasing liver Mn-SOD activity with increasing age, using male and female rats.

The decline in Mn-SOD activity found in this study is not associated with a decrease in the amount of protein (Table 1). Immunoprecipitation experiments showed that the amount of immunoprecipitated Mn-SOD in old tissue is greater than in young tissue extracts. More Mn-SOD protein might mean more mitochondria in the liver with age. However, it has been described that the number of mitochondria decrease with age in liver and heart.[28] Therefore, is not likely that the increase in the amount of Mn-SOD observed could be due to a higher number mitochondria in the tissue with age. The rise in Mn-SOD amount might be due to an accumulation of altered protein in liver during aging.

It is currently assumed that the decrease in activity during aging is due to a post-translational modification of the enzyme by oxidative damage.[29] To test the possible modification by oxidation of Mn-SOD, the amino acid residues most susceptible to oxidation were quantified in young and old Mn-SOD. The results show that there is no difference between the concentrations of cysteine, arginine, lysine and histidine in the Mn-SOD from young and old rats. These results are different from those found in Cu/Zn-SOD, which showed a statistically significant difference between the concentration of histidine residues from young and old rats. However, Mn-SOD is defined as a class separate from Cu/Zn-SOD. The complete amino acid sequence has been determined for both forms from different organisms;[30] the results reported show the absence of any sequence homologies between Cu/Zn-SOD and Mn-SOD. Thus, our results suggest that the accumulation of altered Mn-SOD with age may involve chemical or structural modifications of old Mn-SOD which do not involve the loss of the amino acid residues most susceptible to oxidation.

We have also studied in this paper the behavior with age of total-SOD, Cu/Zn-SOD and Mn-SOD in the lung. The lung is an organ exposed to a greater oxidative stress.[10] Besides, it is one of the extrahepatic tissue with major detoxification capacity.[31] Since the lung sees the highest partial pressure of oxygen in the body (at least at the alveolar membrane), it would be expected that it might experience the greatest oxidative inactivation; however, our findings show that total-SOD, Cu/Zn-SOD and Mn-SOD activities or amount of protein do not present any age-related change in lung (Table 1). Perhaps, the lung might be more efficient in preventing such oxidative damage. Indeed, Frank *et al.*[29] showed that in adult animals of different species, lung antioxidant enzyme activities do not change in response to hyperoxia. Ischiropoulos *et*

al.[32] reported an increase in Cu/Zn-SOD and no variation in Mn-SOD in male rat lung.

Our results indicate that the age-related changes in SOD activity is tissue-specific.

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