Current Topics in Biophysics 2000, 24(2), 39–43

DOES Cu/Zn-SUPEROXIDE DISMUTASE EXHIBIT A NONDISMUTASE ACTIVITY?

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Cu/Zn-superoxide dismutase (SOD) catalyses the dismutation of superoxide anion radical to the nonradical molecules, oxygen and hydrogen peroxide. At the physiological conditions there are defence systems destroying H_2O_2 in the organism such as glutathione peroxidase and catalase. If the removal of H_2O_2 by catalase and peroxidases is insnfficient, the product of the dismutation reaction, hydrogen peroxide can be turned into the substrate for SOD. During this nondismutase activity of Cu/Zn-SOD, copper can be liberated from the active centre of this enzyme. Liberated copper can be chelated with low molecular weight chelators and in this form it can be a substrate for the formation of prooxidant metabolites. In this case, especially when the activity of Cu/Zn-SOD is non-physiologically elevated, it is questionable what the real function of SOD is.

Down syndrome is known to have increased gene dose for Cu/Zn-SOD due to the trisomy of chromosome 21, where the gene for Cu/Zn-SOD is located. We confirmed an increased activity of Cu/Zn-SOD in leukocytes of persons with Down syndrome as compared with the control group. We determined a phenanthroline-detectable low molecular weight copper (chelated with low molecular weight chelators) (LMW-Cu) and we found that LMW-Cu was significantly increased as compared with the controls as well as there was a positive correlation between SOD activity and LMW-Cu in these subjects. On the base of this results we can assume that nondismutase activity of Cu/Zn-SOD can be involved in the potential oxidative stress in persons with Down syndrome.

INTRODUCTION

Cu/Zn-superoxide dismutase (SOD) catalyses the dismutation of superoxide anion radical to the nonradical molecules O_2 and H_2O_2:

$$O_2^{\cdot} + H^+ \xrightarrow{\text{Cu,Zn–SOD}} O_2 + H_2O_2$$

A paradox of this reaction is, that elimination of one oxidant ($O_2^{\cdot}$) causes the formation of next strong prooxidant (H_2O_2). At physiological conditions in our organism there are different defence systems which eliminate hydrogen peroxide. Some of these systems are glutathione peroxidase and catalase (Ďuračková, 1998).

On one hand, at physiological conditions, SOD is the most important intracellular antioxidant, because it eliminates superoxide when it is formed in excess (Ďuračková, 1999).

Only 10 years ago it was believed that SOD plays only a positive role due to its dismutation reaction. *In vitro* studies proved, that SOD eliminates superoxide, inhibits Fe(II) release and inhibits $^{\cdot}OH$ radical formation.

Insufficient activity of SOD *in vivo* is connected for example with the neurodegenerative disease amyotrophic lateral scleroses in which reduction of SOD activity to 50% was determined (Shinobu & Beal, 1998), or with some immunological disorders (Aruoma & Halliwell, 1998).

On the other hand and on the basis of results from the last decade we can not say that the excess of SOD activity is always beneficial *in vivo*. Increased expression of Cu/Zn-SOD can have different negative consequences: (i) increased production of hydrogen peroxide, (ii) reduction of concentration of superoxide which is necessary for its physiological functions, (iii) increased con-

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Abbreviations: SOD, Cu/Zn superoxide dismutase; DS, Down syndrome; TBA, thiobarbituric acid

sumption of metals Cu and Zn followed by inhibition of synthesis of *soxR*, needed for synthesis of proteins involved in DNA repair systems (Liochev & Fridovich, 1992).

When H_2O_2 is in excess Cu/Zn-SOD can be inactivated by hydrogen peroxide with a consequent loss of Cu(II) and $^{\cdot}OH$ formation (Sato, Akaike, Kohno, Ando & Maeda, 1992). Results of other authors exclude free $^{\cdot}OH$ formation after inactivation of SOD by the excess of H_2O_2 and suggest formation of an $^{\cdot}OH$-like entity. This peroxidase-like activity of Cu/Zn-SOD can cause the oxidation of some physiologically important compounds. Increased production of H_2O_2 can also inactivate some glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphatase), initiate HIV gene expression through redox-mediated regulation of transcription factor $NF_{\kappa}B$ (Matthews, Wakasugi, Virelizier, Yodoi & Ray, 1992) and increase of Fe liberation from ferritin. H_2O_2 is a potential substrate for Fenton's type reactions where $^{\cdot}OH$ can be formed.

Increased concentration of H_2O_2 can initiate the peroxidase-like activity of SOD which can result in liberation of Cu(I) from the active site of SOD. It can be a dangerous substrate for the formation of other strong prooxidant copper substances or for hydroxyl radical-like compounds. The evidence for this is the formation of oxo-histidine under these conditions and a peptide chain fragmentation (Jewet, Roclin, Ghanevati, Abel & Marach, 1999). Liberated copper can form complexes with low molecular weight chelators (phosphate, citrate, amino acids, nucleotides, etc.) which can be suitable substrates for Fenton's type reactions.

Except for this, Cu/Zn-SOD can be involved in the nitration of tyrosine residues of proteins by peroxynitrite through the formation of the nitrating agent $NO_2^{+\cdot}$ (Shinobu & Beals, 1998). Nitration of proteins catalysed by Cu/Zn-SOD can have different negative consequences: (i) nitration of proteins is irreversible, (ii) nitrated tyrosine unit of a protein can not be phosphorylated following by inhibition of enzymatic activity, (iii) protein with nitrated tyrosine is more sensitive to proteolytic enzymes.

The potential adverse effect associated with overdoses of SOD in cell-free experimental system was studied by Offer, Russo and Samuni (2000). They concluded that the most significant outcome of higher SOD concentration is a respective decrease in $[O_2^{\cdot}]_{\text{steady state}}$ rather than any significant elevation of $[H_2O_2]_{\text{steady state}}$. At high [SOD] and therefore ultralow $[O_2^{\cdot}]_{\text{steady state}}$, the SOD in its higher oxidation state (Cu(II)/Zn-SOD), not only reacts with $O_2^{\cdot}$, but also attacks and oxidizes the target molecule for which it was supported to provide protection.

Suitable entity for the studying of pathophysiology of increased SOD activity *in vivo* are persons with Down syndrome (DS) (Garaiová, 1999). Their cells are exposed to increase activity of Cu/Zn-SOD caused by trisomy of chromosome #21,where Cu/Zn-SOD is coded. What is the real function of SOD in persons with Down syndrome, where it is believed SOD has 150% activity? Does Cu/Zn-SOD exhibit in these individuals an antioxidant or prooxidant function?

MATERIAL AND METHODS

Sample of patients

Plasma of 20 individuals with Down syndrome, aged from 3 to 20 years. All individuals in this study are regularly monitored at the Down syndrome Department of the Institute for Preventive and Clinical Medicine in Bratislava, Slovakia. For selected examinations, biological samples were taken after a prior written consent of parents.

Individuals with DS suffering from serious heart defects, chronic diseases like diabetes mellitus, children with acute respiration diseases were excluded from the study. The control group was formed by 16 healthy individuals, aged from 7 to 29 years. Blood samples were taken according to the principles of the Helsinki declaration.

Materials

Bovine DNA; 1,10-phenanthroline; ascorbic acid; $CuCl_2 \times 2H_2O$ were from Sigma Chemical Co. (St. Louis, Mo., USA); NaN_3; EDTA, thiobarbituric acid (TBA); trichloroacetic acid (TCA) were from Merck (Darmstadt, Germany). Other chemicals were from Lachema (Brno, Czech Republic). All chemicals used were of p. a. grade.

Determination of low molecular weight copper

Determination of low molecular weight copper (LMW-Cu) was made according to the method of Evans and Halliwell (1994): Liberated copper forms a complex with 1,10-phenanthroline (CuP_2), which is able to cleave DNA. Ascorbate reduces Cu(II) to Cu(I), which reacts with H_2O_2 to form hydroxyl radical. $^{\cdot}OH$ radical cleaves DNA to malondialdehyde which is determined with thiobarbituric acid (TBA).

Total copper in plasma was determined by atomic absorption spectrometry (BM/Hitachi 911).

Isolation of polymorphonuclear leukocytes for SOD determination

Leukocytes were isolated from heparinized venous blood (25 U/ml) using a modification of the Boyum's method in 6% dextrane as published earlier (Muchová, Šustrová, Garaiová, Liptíková, Blažíček, Kvasnička & Ďuračková, in press).

Determination of superoxide dismutase

SOD activity was determined by a photochemiluminiscence method (PCL) using luminol. We used the commercial kit for SOD (F.A.T., Berlin) using bovine Cu/Zn-SOD (Sigma) as a standard. The method is based on a photochemical production of free radicals combined with their photochemiluminiscence detection (Popov & Lewin, 1987) (PHOTOCHEM photochemiluminometer, F.A.T., Berlin). SOD activity is expressed in μg SOD/mg proteins in the cytoplasmic fraction of leukocytes.

Statistical analysis

All results are presented as the mean ± standard error (SEM) along with sample sizes. Between-group comparisons were made using Student's "t" test. We consider as statistically significant those differences, for which the test gave $P < 0.05$. Correlation was expressed using the linear regression correlation coefficient.

RESULTS AND DISCUSSION

The activities of Cu/Zn-SOD in cytoplasmic fraction of leukocytes in DS subjects and controls as well as the concentration of total and phenanthroline-detectable copper (LMW-copper) are given in Table 1.

The difference between the LMW-copper and the total copper in DS and controls is given in Fig. 1. The concentration of copper in controls is assumed as 100%. In DS and controls no difference in the total copper was found while a significantly higher concentration of LMW-copper in DS was determined.

We found a significant positive correlation between Cu/Zn-SOD and LMW-Cu in DS with respect to the control group ($r = 0.476$, vs. 0.07) (Fig. 2).

It is believed that persons with DS are under potential oxidative stress. In our previous work we have found in persons with DS, increased activities of Cu/Zn SOD and GPx in both PMN leukocytes and erythrocytes in agreement with this prediction. However, the activities of catalase, glutathione reductase and myeloperoxidase were not significantly changed (Muchová *et al.*, in press). We asked the question, what can the real reason for the proposal of oxidative stress in DS individuals be. Is it an imbalance in the hydrogen peroxide me-

Table 1. Activity of Cu/Zn-SOD and copper concentration in DS individuals and controls

Parameter	DS	Controls	*p*
Cu/Zn SOD (μg/mg prot.)	8.83 ± 1.80	6.27 ± 1.77	0.001 (23/29)
Total Cu (μmol/l)	17.27 ± 2.58	17.13 ± 3.28	0.85 (23/20)
LMW-Cu (μmol/l)	7.64 ± 4.35	2.59 ± 1.86	0.001 (20/16)

Significance levels *p* is based on between-group (DS vs. controls) comparison using Student's "t" test. Data are given as mean ± SEM (n), n is the number of subjects in a group

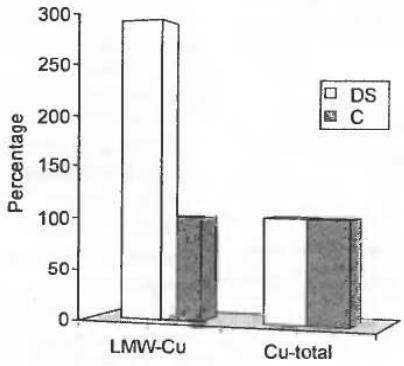

Fig. 1. The level of total and low molecular weight copper (LMW-Cu) in persons with Down syndrome (DS) and controls. The concentration in control is assumed as 100%

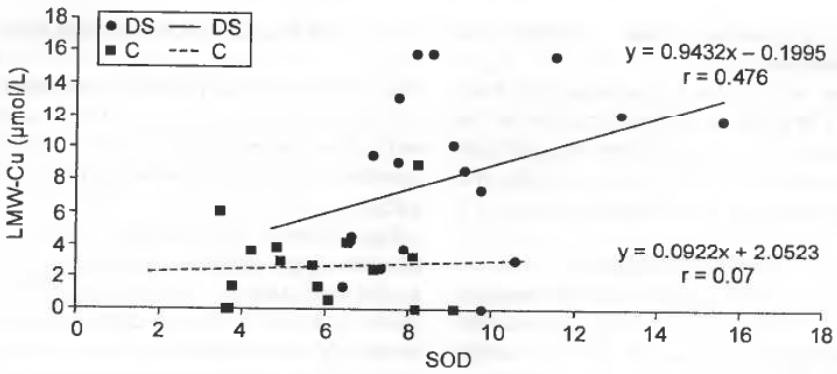

Fig. 2. Correlation between Cu/Zn SOD and low molecular weight copper (LMW-Cu) in Down syndrome patients (DS) (●) and controls (■)

tabolism, which can be formed in higher level by a higher activity of SOD, when catalase activity is not changed, or another unknown factor?

We were interested if Cu/Zn-SOD asserts its hypothetical peroxidase-like activity in DS as proposed in the paper of Jewet *et al.* (1999). We followed it through the determination of LMW-Cu (catalytical active copper), i.e. the copper which is not linked to its physiological chelators. We have found a significantly increased level of catalytical Cu (LMW-copper) determined by the phenanthroline method.

The correlation between LMW-Cu and SOD level in DS in comparison to the controls is very interesting. Elevated LMW-copper as a potential substrate for Fenton reaction or for formation of pro-oxidant Cu-oxygen metabolites (Jewet *et al.*, 1999) could be one of the reason for assumed elevated oxidative stress in DS individuals. For this reason the higher level of LMW-copper found in DS subject supports an assumption of the possible double-sword role of SOD, especially in the case of its increased activity. Whether these "gains of SOD functions" play pathological role in Down syndrome remains the most challenging issue at hand.

Acknowledgement

This research project was supported in part by VEGA grant of Ministry of Education of the Slovak Republic 1/6145/99 and grant of US-Slovak Science and Technology Program 005-96-12. The authors wish to thank to Ms. Ľ. Chandogová and M. Molnárová for their excellent technical assistance.

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