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PARTICIPATION OF METALLOTHIONEIN AND SUPEROXIDE DISMUTASE IN THE BLOOD OF SMOKING SMELTERS

ANNA BIZOŃ and HALINA MILNEROWICZ

Wroclaw Medical University, Wrocław, Poland
Department of Biomedical and Environmental Analyses

Abstract
Objectives: Metallothionein (MT) and two forms of superoxide dismutase (SOD), which are dependent on zinc and copper ions, are involved in defense against the same superoxide anion radicals and are present in extra- and intracellular compartments. The aim of our study was to investigate MT concentration and Cu/Zn SOD activity in the plasma and erythrocyte lysate of the non-smoking and smoking smelters. **Material and Methods:** The investigations were performed in the blood of 300 male smelters and 100 non-exposed male subjects. We have measured zinc, copper, malondialdehyde (MDA) and MT concentrations as well as SOD activity. **Results:** We have observed an increase of Cu/Zn coefficient and decrease of Zn/Cu coefficient in the serum of smelters in comparison with the non-smoking control group. Concentration of MDA in the plasma of smelters was higher in comparison with its concentration in the non-smoking control group. The plasma and the erythrocyte lysate MT concentration increased significantly in the whole group of smelters as compared to the non-smoking control group. The mean value of MT concentration in plasma of the smoking smelters was above 2-fold higher than in the non-smoking control group. The activity of Cu/Zn SOD in plasma of the smoking and non-smoking smelters was decreased in comparison with the smoking and non-smoking control groups, respectively. The lowest activity of Cu/Zn SOD, about 2–3-fold decreased in comparison with the smoking and non-smoking control groups, was detected in plasma of the smelters. An inverse relationship was observed in the erythrocyte lysate. The highest activity of Cu/Zn SOD was reported in the erythrocyte lysate of the smoking smelters and it was about 2-fold higher than in the non-smoking control group. **Conclusions:** In extracellular environment MT plays a crucial role in comparison with the SOD, while in the intracellular compartment Cu/Zn SOD and MT cooperate with each other.

Key words:
Oxidative stress, Cigarette smoking, Occupational exposure, Antioxidants levels

INTRODUCTION

Copper (Cu) is an essential trace element in all living organisms [1]. This metal is found in the prosthetic groups of enzymes that participate in the processes such as cellular respiration, ion transportation and metabolism [2]. As a consequence of its redox properties, excess Cu is potentially toxic to membranes, DNA and protein via Fenton reactions [3]. Superoxide dismutase (SOD) and metallothionein (MT) play an important role in maintaining homeostasis of Cu – they regulate different aspects of Cu uptake, metabolism, traffic, storage and excretion in organism [2]. Moreover, SOD and MT are involved in defense against superoxide anion radicals [$O_2^{\bullet -}$] in extra- and intracellular compartments [4–6]. Additionally, MT

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Corresponding author: A. Bizoń, Wroclaw Medical University, Department of Biomedical and Environmental Analyses, Borowska 211, 50-556 Wrocław, Poland (e-mail: a_bizon@op.pl).

scavenges hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) [7].

SOD is a primary antioxidant enzyme, containing metal ions in active center. There are three main types of SOD, where two of them strictly depend on zinc (Zn) and copper (Cu) – cytoplasmic (Cu/Zn SOD) and extracellular SOD (Ec SOD). The activity of Cu/Zn SOD strictly depends on Zn and Cu, whereas MT participates in the metabolism [3,8].

MT is a low molecular protein and is involved in many physiological and pathophysiological processes such as storage, transportation and metabolism of metal ions and plays a protective role in heavy metal detoxification and against free radicals [9–12]. The role of MT has been extensively studied, but it remains elusive. Partially, the reason is that MT is implicated in multiple physiological processes [2].

It is known that external factors such as heavy metals and tobacco smoking can affect expression of SOD and MT. The deletion of genes encoding SOD or MT can result in disorders in human organisms physiology [13–16]. First, Ghoshal et al. [17] have suggested that the decrease in Cu/Zn SOD activity can cause the increase in MT concentration. This study conducted on Cu/Zn SOD knockout mice has revealed 10–12-fold increase in MT level in the liver as a result of oxidative stress in comparison with the mice with a correct Cu/Zn SOD expression [17]. These results suggest that MT, in the case of lack of expression of Cu/Zn SOD, increases its antioxidant potential as a form of a compensatory mechanism. MT and Cu/Zn SOD can be dependent on or complement each other and these antioxidants are present both in extra- and intracellular compartments. The aim of our study was to investigate MT concentration and Cu/Zn SOD activity in the plasma and the erythrocyte of smoking and non-smoking workers who were exposed to Cu and other heavy metals. This is a second part of our studies conducted in the blood of smelters. First, we have investigated the influence of the intensity of cigarette smoke and duration of occupational exposure in the copper foundry on pro/antioxidant status in the erythrocyte lysate of smelters [18].

MATERIAL AND METHODS

Subjects

The investigations were performed in the blood of 400 subjects: 300 male smelters were recruited from a copper smelting plant in Lower Silesia, in the south-west of Poland (aged 40.8±10.4 years old) and 100 non-exposed male subjects (aged 41.3±6.7 years old). The study protocol was approved by Local Bioethics Committee of Wroclaw Medical University (KB No: 469/2008). Table 1 presents data such as age, body mass index, years of work in the copper foundry industry and smoking habit. The data on smoking, which had been obtained by means of a direct personal interview, were verified by determination of serum cotinine concentrations (DRG® Serum Cotinine EIA-3242, USA). Biological material collected from the control group and the smelter workers was divided into the non-smoking and smoking groups.

Blood collection

The serum was collected according to the routine procedure, by collecting the vein blood to disposable test-tubes. After centrifugation (2500 g / 15 min), the serum was stored at –80°C until marked.

All the blood samples were drawn into trace element-free tubes containing heparin or EDTA and centrifuged at 2500×g for 15 min to separate plasma and buffy coat from the erythrocyte pellet. The pellet was washed in equal volume of ice-cold 0.9% NaCl. This process was repeated twice. The washed cells were lysed by adding ice-cold double distilled water: in the case of heparin 1:1.4 [19] in order to determine the concentration of MT, and in the case of EDTA 1:4 to assay the activity of Cu/Zn SOD. The resulting erythrocyte lysate was used for the assays. Plasma and the erythrocyte lysate were frozen at –80°C until analysis. Concentration of protein in the erythrocyte lysate was determined by the use of the Lowry method [20].

Methods

Measurement of metals concentrations in blood serum was performed by FAAS (flame atomic absorption spectrometry) in an acetylate flame on a SOLAAR M6 (Thermo Elemental, Solaar House, Cambridge, UK) with the appropriate parameters. The accuracy and repeatability of the method were verified by determining metals concentrations in control serum samples (Seronorm TM Trace Elements Serum of Sero AS, Bilingstad, Norway, ref. no. 201405) with known concentrations of the parameters (Cu: 1.19 mg/l, Zn: 1.30 mg/l). The Cu/Zn and Zn/Cu coefficients were also calculated.

Cotinine in serum was measured using the commercial Cotinine ELISA test (DRG® Serum Cotinine EIA-3242, USA).

Concentration of malondialdehyde (MDA) in plasma was measured by tiobarbituric acid (TBA) assay, in which MDA present in plasma, forms a red adduct with two molecules of TBA [21].

Concentration of MT was measured by the two-step direct enzyme-linked immunosorbent assay (ELISA) elaborated in our laboratory using a primary commercial monoclonal antibody Clone E-9 (ref. No: M0639, DakoCytomation, Denmark) and the standard of MT (containing isoforms MT-I and MT-II) isolated from human liver in our laboratory [22].

Cu/Zn SOD activity was determined with Superoxide Dismutase Assay Kit (ref. No: CM706002, Immuno-Biological Laboratories, Germany).

Statistical analysis

The data are expressed as mean (SD) values. The normality of the variable was tested by the Shapiro-Wilk W test. Differences between the groups were tested using Student's t test with an equal variance assumption (Zn, Cu, cotinine in serum, MDA in plasma, MT and SOD in plasma and in erythrocyte lysate). Correlation was expressed by the Pearson correlation coefficient (r). In all instances, $p < 0.05$ was considered statistically significant. Statistical calculations were performed using the STATISTICA version 9.0 (Polish version; StatSoft, Kraków, Poland).

RESULTS

Concentration of cotinine in serum

Investigations demonstrated a statistically significant increase in the levels of cotinine – nicotine metabolite in the serum of the smoking smelters and smoking men from the control group, which confirms the data previously obtained from a direct personal interview. The average cotinine level in the serum of the non-smoking groups indicated lack or minor exposure to tobacco smoke (Table 1).

Concentration of Cu in serum and the value of Cu/Zn coefficient

The Cu level was increased in all groups of smelters in comparison with the Cu level in the non-smoking control

Table 1. Baseline characteristics of the unexposed non-smoking and smoking control subjects and smelters

Variable	Control group (M±SD)		Smelters (M±SD)	
	non-smoking (N = 42)	smoking (N = 58)	non-smoking (N = 122)	smoking (N = 178)
Age (years)	37.15±11.26	39.64±11.49	45.09±8.95	41.92±9.27
BMI	25.61±3.44	27.36±5.68	27.56±3.64	26.12±3.81
respondents with BMI ≥ 25 (n)	22	40	88	89
Cigarettes (n/day)	–	16.57±4.64	–	18.22±9.88
Cotinine concentration (ng/ml)	1.65±1.00	72.65±21.87*	2.20±1.86	83.24±20.64**

BMI – body mass index; M – mean; SD – standard deviation.
* Significant ($p < 0.01$) when compared to non-smoking control group.
** Significant ($p < 0.01$) when compared to non-smoking smelters.

Table 2. The concentrations of Cu, Zn and value of Cu/Zn coefficient as well as the selected oxidative stress parameters of the non-smoking and smoking control subjects and smelters

Variable	Control group (M±SEM)		Smelters (M±SEM)	
	non-smoking (N = 42)	smoking (N = 58)	non-smoking (N = 122)	smoking (N = 178)
Cu in serum (mg/l)	0.97±0.11*,**	1.04±0.13	1.03±0.15*,#	1.07±0.16**,#
Zn in serum (mg/l)	0.99±0.11	0.98±0.09	0.95±0.16	0.95±0.15
Cu/Zn	1.00±0.17*,**	1.07±0.18	1.11±0.19*	1.16±0.22**
Zn/Cu	1.06±0.16*,**	0.99±0.11	0.94±0.17*	0.90±0.18**
MDA in plasma (μmol/l)	2.02±0.66*,**,***	3.00±0.91*	3.20±1.04**	3.48±1.23***
MT in lysate (μg/g protein)	14.08±2.03*,**	15.98±3.42	16.54±3.66*	20.55±14.37**
MT in plasma (ng/ml)	2.81±1.51*,**	4.21±2.11	4.96±2.20*	5.74±3.24**
Cu/Zn SOD in lysate (U/g protein)	86.61±34.83*	102.88±54.69	136.22±76.43*,#	151.06±61.81#
Cu/Zn SOD in plasma (U/ml)	31.27±9.01*	31.58±9.01**	11.44±5.58*,#	14.88±8.22**,#

Cu – copper; Zn – zinc; Cu/Zn – Cu/Zn coefficient; Zn/Cu – Zn/Cu coefficient; MDA – malondialdehyde; MT – metallothionein; Cu/Zn SOD – copper-zinc superoxide dismutase.
M – mean; SEM – standard error of mean.
*, **, *** Significant ($p < 0.05$) when compared to non-smoking control group.
Significant ($p < 0.05$) when compared to non-smoking smelters.

group. The highest concentration of Cu was detected in serum of the smoking smelters. The values of Cu/Zn coefficient in serum of smelters were increased in comparison with these values in the non-smoking control group (Table 2).

Concentration of Zn in serum and the value of Zn/Cu coefficient

No significant difference was noted between the concentration of Zn in serum of the control groups and the smelters. The values of Zn/Cu coefficient in serum of the smelters were decreased in comparison with the non-smoking control group (Table 2).

Concentration of MDA in plasma

Concentration of MDA in plasma of the smelters was statistically higher in comparison with that of the non-smoking control group. Tobacco smoking was an additional factor that caused elevation of MDA concentration (Table 2).

Concentrations of MT in plasma and erythrocyte lysate

MT concentration in plasma and the erythrocyte lysate increased significantly in all groups of smelters as compared with the non-smoking control group. The highest concentrations of MT both, in plasma and the erythrocyte lysate, were observed in the smoking smelters. The mean value of MT concentration in plasma of the smoking smelters was above 2-fold higher than in the case of the non-smoking control group (Table 2).

Activities of Cu/Zn SOD in plasma and erythrocyte lysate

The activity of Cu/Zn SOD in plasma of the smoking and non-smoking smelters was significantly decreased in comparison with the smoking and non-smoking control groups, respectively. The lowest activity of Cu/Zn SOD, about 2–3-fold decreased in comparison with the smoking and non-smoking control, was detected in plasma of the smelters. An inverse relationship was observed in the case of erythrocyte lysate. The highest activity of Cu/Zn SOD

Table 3. Correlations between Cu, MDA, MT concentrations and activity of Cu/Zn SOD in the blood of smelters

Correlations (N = 300)	Cu/Zn SOD		MT	
	in plasma (U/ml)	in erythrocyte lysate (U/g of protein)	in plasma (ng/ml)	in erythrocyte lysate (µg/g of protein)
Cu in serum (mg/l)	ns	r = 0.68 p = 0.00	r = 0.23 p = 0.03	r = 0.28 p = 0.03
MDA in plasma (µmol/l)	r = –0.65 p = 0.00	r = 0.30 p = 0.03	r = 0.51 p = 0.00	r = 0.49 p = 0.01

ns – non significant.
r – Pearson correlation coefficient.
Other abbreviations as in Table 2.

Table 4. Correlations between MT concentration and Cu/Zn SOD activity in the blood of smelters

Correlations (N = 300)	Cu/Zn SOD in plasma (U/ml)	Cu/Zn SOD in lysate (U/g of protein)
MT in plasma (ng/ml)	r = –0.35 p = 0.01	–
MT in lysate (µg/g of protein)	–	r = –0.46 p = 0.01

Abreviations as in Table 2.

was reported in the case of erythrocyte lysate of the smoking smelters and it was about 2-fold higher than that in the non-smoking control subjects (Table 2).

Correlations

Occupational exposure to Cu and tobacco smoke cause an elevation of MDA concentration and induced enzymatic and non-enzymatic antioxidants in the erythrocyte lysate, which is manifested by the increased MT concentration and Cu/Zn SOD activity (Table 3). We have observed a negative correlation between MT concentration and Cu/Zn SOD activity in the erythrocyte lysate and in plasma (Table 4).

DISCUSSION

MT modulates the activity of Zn- and Cu-dependent metalloenzymes such as Cu/Zn SOD [23]. Both, MT and Cu/Zn SOD remove free radicals in extra- and intracellular environment. It was observed that Cu/Zn SOD knockout mice have revealed 10–12-fold increase in MT level in the liver as a result of oxidative stress in comparison with the mice with a correct Cu/Zn SOD expression [17]. The higher elevation of MT concentration was explained by an increase in $O_2^{\bullet -}$ accumulation, which can induce MT synthesis. The lack of Cu/Zn SOD affects higher concentration of free Zn^{+2}, which can induce MT expression [17]. In a previous study we have observed a positive correlation between MT concentration in erythrocyte lysate and cadmium and lead concentrations in the whole blood and Cu/Zn SOD activity in erythrocyte and lead concentration in the whole blood [18]. In the current study we have measured concentration of Zn, Cu, MT and the Cu/Zn SOD activity as the elements engaged in all the essential processes in extra- and intracellular compartments in blood.

It was confirmed that higher Cu concentration can result in disorders of Zn homeostasis. Also exposure to heavy metals such as cadmium and lead present in copper foundry can disturb Cu and Zn homeostasis [24–26]. Cd can displace Zn or Cu revealed by a decrease in Cu and Zn levels in the enzymes and an increase of these two metals in the cytoplasm. This results in conformational changes and inhibition of enzyme activity (Cu/Zn-SOD for example) [26].

In the present study we have observed a higher value of Cu/Zn coefficient and a lower value of Zn/Cu coefficient in the serum of the non-smoking and smoking smelters. The disorders of Cu/Zn and Zn/Cu values in the serum of the smelters probably result from the increase in Cu concentration as an effect of occupational exposure and decrease in Zn concentration. The reduced concentration of Zn can result from the disturbances in albumin synthesis, improper absorption, impairment of ileo-pancreatic circulation and hyperglycemia [27].

The levels of Cu and Zn can depend on Cu/Zn SOD activity and MT concentration. An elevation of MT concentration in plasma can result from a higher accumulation of Cu. In plasma of the smelters, where the concentration of MT was the highest, activity of Cu/Zn SOD was the lowest. We have also observed a negative correlation between MT and Cu/Zn SOD ($r = –0.35$; $p = 0.01$), which suggests the crucial role of MT in plasma as an antioxidant.

Considering the expressions of MT and Cu/Zn SOD in the intracellular spaces we have observed a higher erythrocyte lysate MT concentration and Cu/Zn SOD activity. However, the increase in Cu/Zn SOD activity was much higher in comparison with the control group (about 2-fold higher in the erythrocyte lysate of the smoking smelters than in the erythrocyte lysate of the non-smoking control group) than MT concentration. MT and Cu/Zn SOD remove the same free radical – $O_2^{\bullet -}$, but Cu/Zn SOD scavenges $O_2^{\bullet -}$ almost 2-fold faster than MT, which can also explain higher Cu/Zn SOD activity in the erythrocyte lysate of the smelters.

Miyayama et al. have investigated the expressions of genes encoding Cu/Zn SOD and MT [1]. In their research, small interfering RNA (siRNA) targeting copper chaperone for SOD1 (CCS) was introduced into MT-knockout mice fibroblasts (MT-KO cells) and their wild type cells (MT-WT cells) to reveal the interactive role of CCS with other Cu-regulating proteins, in particular MT [1]. Their results have shown that in CCS-knockdown cells MT can be a donor of Cu [1]. However, the role of MT as a donor of Cu in the lack of CCS is still unclear. It was also demonstrated that MT binds Cu and is able to deliver Cu to apo-SOD [8]. CCS knockdown in MT-WT cells of fibroblasts induces expression of MT isoforms, which probably is related to the presence of free Cu. However, activity of Cu/Zn SOD was not affected by the transfection of CCS-targeting siRNA in both, wild type mice and MT knockout mice. This suggests that remaining amount of CCS could be sufficient to deliver Cu to Cu/Zn SOD1. Accumulation of Cu in the cells of transgenic mice induces synthesis of MT and protects against toxicity of free Cu ions, which also suggests that indirect MT is involved in the regulation of Cu/Zn SOD expression and activity [1].

The study conducted by Levy et al. on mice with overexpression and quenched Cu/Zn SOD coding genes has also confirmed the important role of this enzyme in Cu homeostasis in the liver and proved the simultaneous increase in Cu/Zn SOD activity and Cu ions level [28].

In the present study in serum of the smelters, in whom the highest increase of Cu concentration was observed, Cu/Zn SOD activity in erythrocyte lysate was the highest. A positive correlation ($r = 0.68$; $p < 0.001$) between Cu concentration in serum and Cu/Zn SOD activity in erythrocyte lysate of the smelters was noted, which confirms the relationship between these markers.

Similar results were obtained by Tapia et al., who also suggest, that compensatory mechanism of MT and Cu/Zn SOD can result from changes of Cu status. The study was conducted on cell line from embryos of mice with the deletion of genes encoding MT and wild type mice. The cell cultures

were incubated with medium containing isotope ^{64}Cu. MT mutant cells were more sensitive to an increased intracellular content of Cu and had lower viability than wild type fibroblasts [2]. These results support the role of MT in detoxification of Cu, in regulation of intracellular Cu level during adaptation to extracellular Cu excess and as a reservoir of Cu to be used for cuproenzymes synthesis such as Cu/Zn SOD in the case of Cu deficiency [2].

Our earlier studies have also suggested that these antioxidants can interact with each other. Investigation conducted in the blood of the patients with acute pancreatitis (AP), chronic exacerbated pancreatitis (CEP) and chronic pancreatitis (CP) has shown different changes of MT and Cu/Zn SOD expression depending on the kind of pancreatitis [29]. MT was a specific marker for acute inflammation while Cu/Zn SOD was characteristic for chronic diseases [29]. The results of immunohistochemical studies also support the essential and different roles of MT and Cu/Zn SOD in the inflammatory processes in pancreas. Elevated expression of MT in pancreatic vesicular cells and islets can confirm the role of this gland in the storage of Zn ions as well as its protective function in the struggle with ROS in this system of pancreatic cells [29].

Differences in the status of Cu/Zn SOD and MT can depend on tobacco smoking, age or weight. It was clearly shown that the increase in age affects the elevation of MT concentration [30], while the activity of Cu/Zn SOD a decrease in it [31]. Also heavy metals accumulated in proportion to years of exposure can induce the synthesis of MT [23].

The decrease in Cu/Zn SOD activity can be caused by irreversible inactivation by the final product of reduction reaction of $O_2^{\bullet-} - H_2O_2$ [32]. The increase in MT concentration and decrease in Cu/Zn SOD dependent on ageing also suggest that these antioxidants can complement each other. However, this section of the study needs further attention.

In conclusion, we have observed the crucial role of MT in extracellular environment. In intracellular compartment Cu/Zn SOD and MT cooperate with each other.

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