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## Changes of Metallothionein, Copper, Zinc, and Zinc-dependent Enzymes Induced by Immobilization Stress<sup>#</sup>

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In order to study the effects of stress on copper (Cu), zinc (Zn), Zn-related enzymes, and metallothionein (MT) in liver, we used the immobilization procedure as an experimental model of stress. Acute immobilization stress caused changes of Zn, Cu, and MT concentrations in rat liver. The Zn level in total liver homogenates increased for 39% ( $p < 0.00011$ ). This increase originated from the Zn concentration in nuclear fraction, suggesting a higher content of Zn-dependent enzymes and large molecular weight proteins. The stress induced increase of alkaline phosphatase (ALP), which is Zn-related metalloenzyme, closely followed the changes in the Zn concentration in liver. However, the activity of another Zn-dependent enzyme, 5'-nucleotidase (5'-NT), was lower after acute stress. The total Cu concentration was about twice higher in the stressed group in comparison with the control group, but no redistribution of Cu within hepatocytes was found. MT concentration was measured in the mitochondrial-lysosomal fraction of liver homogenates. It was found that acute immobilization stress caused about a 100% increase in the MT concentration in liver.

### INTRODUCTION

Oligoelements, copper (Cu) and zinc (Zn), are essential for a variety of biological functions in the organism. Cu is required for bone formation, proper cardiac function, connective tissue development, myelination of the

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spinal cord, keratinization and tissue pigmentation.<sup>1-3</sup> Cu is involved in these processes *via* Cu-metalloenzymes. In most cases, the function of Cu in metalloenzymes involves electron transfer and enzymatic binding of molecular oxygen. Some of Cu-dependent metalloenzymes are: ceruloplasmin, cytochrome c-oxidase, dopamine  $\beta$ -hydroxylase, monoamine oxidase, superoxide dismutase (SOD), lysyl oxidase, *etc.* SOD is an extremely important and well-studied Cu-metalloenzyme, abundant in the liver as well as in other tissues.<sup>4</sup> It catalyzes dismutation of the superoxide anion and closely follows the Cu concentration. Another Cu-dependent metalloenzyme, lysyl oxidase, is important in the biosynthesis of connective tissue.<sup>5</sup> Its activity in the liver is related to collagen synthesis during hepatic fibrosis.<sup>6</sup>

The multiplicity of Zn functions is due to its role in specific metalloenzyme systems. Zn is a constituent of about 120 enzymes, among which are alkaline phosphatase (ALP), carboxypeptidase, oxido-reductase, carboanhydrase, transferase, liase, isomerase, DNA and RNA polymerase, reverse transcriptase, *t*RNA synthetase, and elongation factors.<sup>7</sup> The presence of Zn is required for bone formation, cell-mediated immunity, generalized host defense, and a variety of factors related to tissue growth. Zn-finger loop is present in the DNA binding domain of glucocorticoid-, mineralocorticoid-, estrogen-, progesterone-, androgen-, thyroid-, vitamin D, and retinoic acid-receptors.<sup>8-10</sup> Depletion of Zn from such domains could be involved in endocrine manifestation of Zn deficiency.

The most important role in the metabolism of trace elements, particularly, Cu and Zn, seems to be played by metallothionein (MT), a highly conserved cysteine rich single polypeptide chain with the metal-binding capacity of 5 to 7 g atoms/mol.<sup>11</sup> Although MT has been the most widely studied metalloprotein, its exact function(s) remains to be defined. The fact that MT binds Cu and Zn under physiological conditions suggests that the protein is involved in the metabolism of both nutrient metals. The binding phenomenon is responsible for maintaining the Zn and Cu homeostasis.<sup>12</sup> However, other MT functions are possible. A role as a donor of Zn and Cu ions to apometalloproteins has been proposed on the basis of *in vitro* data. There is correlation between MT induction and host defense that suggests that donation of bound metals to metalloproteins is a possible function of MT within the framework of a host-defense process.<sup>13-15</sup> The next very important function of MT is detoxication of heavy metals.<sup>16</sup>

Synthesis of MT is controlled by complex processes. MT gene expression is regulated by metals that bind to proteins. It has been demonstrated that Zn, Cu and cadmium (Cd) induce MT.<sup>17,18</sup> There are many other inducers of MT synthesis, among which are glucocorticoids, glucagon, and epinephrin.<sup>19-21</sup> Hormonal regulation of MT synthesis and the important role of MT in the trace element metabolism suggest that MT could be an important link between microelements and hormonal action, characteristic of stress response.

The aim of our study was to study how the acute stress condition affects Cu, Zn, Zn-related metalloenzymes, and MT in liver. For this purpose, we have used the immobilization stress procedure as an experimental model of acute stress.

## MATERIAL AND METHODS

### *Subjects*

A group of four months old Wistar rats ( $N = 11$ ) was immobilized for three hours in order to create the condition of acute stress. The control group ( $n = 6$ ) was deprived of water and food during that time. The animals were sacrificed 24 hrs after stress treatment, and the livers were rapidly removed.

### *Cell fractionation*

Homogenates were prepared in 0.14 M KCl with Teflon pestle in a glass homogenizer. Distinct cell fractions were prepared by differential centrifugation at 4 °C. The crude nuclear fraction (nuclei and cell debris) was obtained by centrifuging the homogenate at 1 400 g for 5 min. The resulting supernatant was then centrifuged at 21 000 g for 20 min, yielding the pelleted mitochondrial-lysosomal fraction and supernatant – the post mitochondrial fraction (cytosol and microsomes).

### *Determination of Zn, Cu and MT*

Concentrations of Zn and Cu were determined in total liver homogenates, as well as in nuclear (1 400 g pellet), mitochondrial-lysosomal (21 000 g pellet) and cytosol-microsomal fractions (21 000 g supernatant). For the analysis atomic absorption spectrometry (AAS) of pretreated acidified samples according to the method of Luterotti *et al.*, was applied.<sup>22</sup>

MT concentration was measured in 21 000 g supernatant fraction using Cd-hem method.<sup>23</sup>

### *Alkaline phosphatase (ALP) and 5'-nucleotidase (5'-NT) assay*

ALP (E. C. 3.1.3.1.) was measured in the plasma-membrane fraction (18000 g pellet) using the standard assay.<sup>24</sup>

5'-NT (E. C. 3.2.3.5.) was also measured in the plasma-membrane fraction of liver homogenate in the presence of phosphatase inhibitor levamisol in a concentration of 1 mM. The assay is based on determination of Pi released in the reaction of 5'-NT with its substrate AMP. Pi was determined according to Fiske and Subbarow.<sup>25</sup>

### Protein determination

Protein concentrations were estimated according to Lowry.<sup>26</sup>

### Statistical data analysis

Data analysis was performed with the computer software "Statgraphics™" (STSC Inc. and Statistical Graphics Corp., Los Angeles, USA) using the *t*-test and the analysis of variance (ANOVA one way).

## RESULTS

### Cu, Zn and MT

Cu concentrations were significantly increased ( $p < 0.0004$ ) in total liver homogenates of stressed animals in comparison to the Cu content in the control samples, as shown in Table I. This increase is a cumulative effect of elevated Cu concentrations in two different cell fractions, *i.e.* in the 1 400 g and 21 000 g pellet fractions. The absolute rise in the amounts of Cu in stressed samples of the whole homogenate could be accounted for homogenate by proportionally higher Cu concentrations in the nuclear and mitochondrial-lysosomal fractions, thus no redistribution of Cu within hepatocytes was noticed after acute stress.

TABLE I

Copper content in liver homogenates and cell fractions of stressed ( $n = 11$ ) and control ( $n = 6$ ) animals

Liver fraction	Copper content μg/g dry wt. ± SD		Statistical significance <i>p</i> < <i>t</i> -test (ANOVA one way)	Difference % of the control value
	stress sample	control		
Homogenate	60.4 ± 12.5	32.8 ± 6.1	0.00004 (0.0002)	+84
1 400 g pellet	33.5 ± 8.5	17.4 ± 4.4	0.0002 (0.001)	+93
21 000 g pellet	14.4 ± 7.6	7.1 ± 3.3	0.034 (0.059)	+103
21 000 g supernatant	9.7 ± 3.4	7.6 ± 3.4	0.295 (0.280)	+28

Acute stress caused a significant increase of the Zn level in the total liver homogenate, arising from the elevated Zn concentrations in nuclear and mitochondrial-lysosomal fraction of hepatocytes. The data are shown in Table II.

TABLE II

Zinc content in liver homogenates and cell fractions of stressed ( $n = 11$ ) and control ( $n = 6$ ) animals

Liver fraction	Zinc content $\mu\text{g/g dry wt.} \pm \text{SD}$		Statistical significance $p <$ $t$ -test (ANOVA one way)	Difference % of the control value
	stress sample	control		
Homogenate	$188.8 \pm 14.4$	$135.9 \pm 3.3$	0.00001 (0.00011)	+39
1 400 g pellet	$84.5 \pm 17.5$	$37.8 \pm 5.1$	0.0004 (0.0007)	+123
21 000 g pellet	$55.9 \pm 20.6$	$44.5 \pm 17.4$	0.305 (0.433)	+26
21 000 g supernatant	$51.5 \pm 15.3$	$53.5 \pm 5.3$	0.717 (0.775)	-4

About 52% of liver Zn was found in the 1400 g pellet in animals exposed to immobilization stress, while the control group contained only about 33% of total Zn in the corresponding fraction. This result suggests that the acute stress induces not only Zn accumulation in liver, but also, an overload in the nuclear compartment.

In the 21 000 g supernatant fraction of liver homogenates obtained from stressed animals MT was found to be  $123 \pm 44 \mu\text{g/g dry mass}$ . In the control group,  $57 \pm 18 \mu\text{g/g dry mass}$  of MT was found. A significant increase ( $p < 0.094$ ) of MT concentration could be identified as a consequence of acute stress.

#### *Alkaline phosphatase and 5'-nucleotidase*

The catalytic activity of ALP in the plasma-membrane fraction of the liver from rats exposed to immobilization stress was  $138 \pm 36 \text{ U/g}$ . The ALP activity in the control group was  $60 \pm 11 \text{ U/g}$ . Thus, a significant increase ( $p < 0.00008$ ) of ALP activity occurred after acute stress.

Comparison of 5'-NT activity in the plasma-membrane fraction of the control group ( $694 \pm 155 \text{ U/g}$ ) and stressed animals ( $438 \pm 77 \text{ U/g}$ ) shows a decrease in enzymatic activity related to acute stress.

## DISCUSSION

Experimental acute stress, created through immobilization procedure, affected Cu, Zn and MT concentrations as well as the ALP and 5'-NT activity in liver.

Hepatic Zn concentration increased significantly in stressed rats. This rise of total Zn concentration is closely followed by an increase of Zn-level in the nuclear fraction, suggesting that the stress induced increase of Zn should be attributed to non-metallothionein proteins rather than to the accumulation of Zn in the MT fraction in liver.

The measured activity of ALP closely followed elevation of Zn the concentration caused by stress. ALP acts on a large variety of physiological and non physiological substrates; the nature of the precise metabolic function of the enzyme is not yet understood. It appears, however, that the enzyme facilitates transfer of metabolites across cell membranes, and that it is associated with lipid transport.<sup>27</sup> Since the exact function of ALP is not defined, it is unclear whether the increased activity could be understood in the context of the defense mechanism to stress.

The other Zn-dependent enzyme, 5'-NT, was found to be less active after acute stress. There are at least two possible explanations for a decreased 5'-NT activity: the decrease is either due to the inhibition of 5'-NT on the gene level or it is due to the inhibition of the enzyme activity by the observed increase of inhibitor concentrations, *i.e.* Zn and Cu. 5'-NT catalyzes the extracellular dephosphorylation of purine and pyrimidine ribonucleoside and deoxyribonucleoside 5'- monophosphate. Although no single physiological role has been assigned to the enzyme, several have been suggested, including nucleotide scavenging or production of adenosine as a "local hormone".<sup>28</sup> Nucleotide scavenging and/or production of adenosine have to be suppressed after acute stress, but whether this suppression takes place in stress response is still unclear.

Cu concentrations are strongly increased in total cell homogenates following the elevation of Cu levels in nuclear and mitochondrial-lysosomal fractions. Thus, no stress related redistribution of the Cu content within cell compartments was observed. Cu is very toxic to hepatocytes.<sup>29</sup> There are possibilities, however, to protect liver against Cu toxicity, *e.g.* by its binding to MT, and by intracellular transfer into the mitochondrial-lysosomal fraction.<sup>30</sup> It is unclear whether this mechanism takes place in preventing the liver damage after acute stress because no significant redistribution of Cu was detected in the hepatocytes of stressed animals.

MT plays the central role in the metabolism of heavy metals. It is biosynthetically regulated at the level of gene transcription in response to metals, hormones, cytokines and other physiological and environmental stimuli.<sup>19-21,31</sup> Its physiological function is not fully understood. It is generally accepted that the principal roles of MT lie in detoxification of heavy metals and regulation of the metabolism of essential trace elements. However, there is increasing evidence that it can act as a free radical scavenger protecting the cell against oxidative damage.<sup>32</sup> The immunomodulatory function of extracellular MT was also suggested.<sup>33</sup> Taken together, MT could be a part of the general protective system in a mammalian cell.

Induction of MT after acute stress points to the important function of this protein in the defense mechanism of a living organism to stress conditions. Thus, the stress affected Zn and Cu concentrations, being closely related to many essential biological processes, among which is the immune response, substantially contribute to the warning of stress related dangers and open a new area of research to elucidate more clearly the present findings.

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**SAŽETAK****Koncentracijske promjene metalotioneina, bakra, cinka i cink-ovisnih enzima u imobilizacijskom stresu**

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Proučavan je utjecaj akutnog stresa na sadržaj bakra (Cu), cinka (Zn) i metalotioneina (MT) u štakorskoj jetri. Trosatni imobilizacijski stres uzrokovao je statistički značajan porast Zn ( $p < 0,00011$ ) za 39% u jetrenom homogenatu životinja koje su pretrpjele stres. Taj je porast rezultat povišenja Zn u nuklearnoj frakciji, to upućuje na povećanje koncentracije enzima ovisnih o Zn, kao i Zn-proteina velike molekularne mase u staničnoj jezgri. Mjerene su aktivnosti dvaju enzima ovisnih o Zn, alkalne fosfataze (ALP) i 5'-nukleotidaze (5'-NT) u frakciji plazmatskih membrana. Aktivnost ALP slijedi promjene ukupne koncentracije Zn u jetri, dok je 5'-NT snižena. Slično Zn, ukupna koncentracija Cu viša je oko 2 puta u jetrama životinja koje su pretrpjele stres, u usporedbi s kontrolnima. Nije zamijećena redistribucija Cu u stanicama jetre kao posljedica akutnoga stresa. Koncentracija MT je mjerena u citosolno-mikrosomalnoj frakciji jetrenog homogenata. Akutni je stres uzrokovao oko 100% povišenje koncentracije metalotioneina u jetri.