UC 577,1; 61 ISSN 0354-3447

Jugoslov Med Biohem 23: 1–9, 2004

Pregledni članak
Review article

CELLULAR STRESS RESPONSE – DEFENCE AGAINST METAL TOXICITY

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Summary: All cells respond to various types of stress by increasing the transcription of specific genes that encode class of proteins termed stress proteins. This response is believed to represent a transient reprogramming of gene expression and biological activity, which serves to protect sensitive cellular components from damage, and assists in the rapid recovery after the stress is removed or ceases. The synthesis of stress proteins can be induced under a host of different stress conditions, including elevated level of metals. Although, understanding of the relationships between metals and their capacity to induce stress response is incomplete, these interactions are important to consider because they may reveal information regarding mechanisms of toxicity, cellular defence mechanisms against metal toxicity, and biochemical responses which can be exploited as biomarkers of exposure and toxicity of metals. This review is focused on two main classes of stress proteins, metallothioneins (MTs) and heat shock proteins (Hsps), which are usually induced in response to stress provoked by metals. It summarizes the results of studies on metals toxic effects and their ability to induce cellular stress response.

Key words: metals, cellular stress response, metallothionein (MT), heat shock protein (Hsp)

Introduction

Nearly three-quarters of the elements that make up the universe are metals. Though they are abundant in nature, and though many are essential for life, some metals can be toxic to living things when they build up in water, soil or food. Released into the biosphere by human activity, metals, unlike organic chemicals, do not break down. There is a growing awareness of the extent and complexity of this problem worldwide. How living cells cope with this problem? The cells under unfavourable conditions activate detoxification mechanisms to protect themselves from harmful effects of various stressors including toxic metals.

The aim of the present review is to provide better understanding of the molecular basis of metal toxicity and cellular ability to cope with problem of metal intoxication. Special attention will be focused on two main classes of proteins, metallothioneins (MTs) and heat shock proteins (Hsps), which perform the main role in cellular stress response to metals and in their detoxification.

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Molecular basis of metal toxicity

Metal ions form an essential feature of the human environment. Some are important in nutrition as trace elements; others are toxic for one or more cell types, organs or organisms. Metals can have a variety of physiological effects and it is often possible to demonstrate the toxicity of any given metal in any given organ, provided that the dose is high enough and prolonged enough. Essential metals (with known biological function) may be toxic at a dose that overwhelms homeostatic controls over absorption and excretion. Also, essential metals in certain forms can be toxic. For example, chromium in the form of Cr^{3+} is an essential trace element important for maintaining correct blood sugar levels, but in the form of Cr^{6+} is a known human lung carcinogen (1).

The nonessential, toxic metals (with unknown biological function) are cadmium (Cd), mercury (Hg),

lead (Pb) and metalloid arsenic (As). Actions of these metals include substituting for essential elements in enzymatic reactions, energy metabolism, neurotransmission, or structural components (bone) (2). Toxic metals can react covalently or noncovalently with enzymes, membranes and DNA, or can stimulate the production of reactive oxygen species (3). The variety of toxic effects makes it difficult to determine which of the actions is responsible for toxicity of a metal in its target tissue. Metals can interact with each other to enhance toxicity (e.g. by affecting the same target organs), or to reduce toxicity (e.g. by stimulating defence mechanisms) and this must be carefully considered when interpreting experimental results (4). Further, in determination of toxic effects of a metal, many different factors, such as chemical form of the metal, route and duration of exposure, age at exposure, possibility of the metal absorption, distribution and excretion, and other factors have an important role (5).

Based on their reactivity, metals are divided into two groups. The first group comprises redox-active metals, such as iron (Fe) and copper (Cu), that mediate production of reactive oxygen species (ROS). The second group includes redox-inert metals, such as Cd, Hg, Pb, As and zinc (Zn), which do not participate in direct production of ROS. Metals belonging to the latter group have high affinity for sulphydryl groups, especially for protein thiols.

Redox-active metals

A growing body of evidence indicates that transition metals act as catalysts in the oxidative deterioration of biological macromolecules, and therefore, the toxicity associated with these metals may be due, at least in part, to oxidative tissue damage (3).

The basic mechanisms of ROS production by transition metals involve Fenton/Haber-Weiss chemistry and autooxidation (6). In these reactions molecular oxygen is activated to superoxide anion radical during autooxidation, and hydrogen peroxide activated to hydroxyl radical in the presence of metal ions through Fenton/Haber-Weiss chemistry (7). Hydroxyl radical is a species frequently proposed to initiate membrane lipid peroxidation.

The toxicity produced by redox-active transition metals generally involves hepatotoxicity, nephrotoxicity and neurotoxicity (6). Specific differences in the toxicity of metal ions may be related to differences in solubility, absorbability, transport, chemical reactivity, and the complexes that are formed within the body. Many human diseases are connected with imbalance of metal ions homeostasis, and develop as a consequence of disrupted metal absorption, transport or excretion, which lead to excessive free metal ions accumulation in tissues. Typical examples are Menkes and Wilson diseases, which are caused by defect in Cu transport (8). Also, several neurodegenerative diseases, such as Alzheimer disease, prion disease and familiar amyotrophic lateral sclerosis, are connected with elevated ROS production on account of disturbed balance of Cu, Fe and Zn (9).

Redox-inert metals

The toxic effects of the most harmful metals can be traced to their ability to disrupt the function of essential biological macromolecules. Toxicity of Cd, Hg and Pb in the organism is usually realised by their ability to replace essential metals from enzymes or other proteins in the cell, or to interact with thiol groups of proteins (2).

Substitution of essential metal in the active centre of an enzyme (10), or in a Zn-finger transcription factor (11) with a toxic metal, provokes alterations in the structure and function of the protein, and in accordance with it, a series of unwanted consequences. At least eight to ten proteins, involved in the nucleotide excision repair mechanisms, could be inactivated in the presence of metal ions, such as Cd, Pb, Co and Ni (10), which could elevate the number of mutations and thus, the risk for carcinogenesis. It is well documented that Cd interacts with the metabolism of Zn and Ca, and also, with Fe and Cu metabolism (4, 12). Further, influence of Pb on Ca or Zn metabolism is important part of Pb toxicity, with the adverse consequences on nervous, hematopoetic, renal and skeletal system, and especially on CNS as the primary target organ (4, 12). Our previous results pointed out to ability of Pb to inhibit the activity of d-aminolevulinic acid dehydratase (the key component of the heme biosynthesis pathway) in murine erythrocytes and liver via competition with Zn (13). This enzyme is a good biomarker of exposure for organisms intoxicated with Pb. Besides, Hg could decrease the cellular protection against oxidative stress due to its ability to interfere with selenium (Se)-dependent enzymes involved in glutathione biosynthesis (14).

Because of their high reactivity with thiol groups, the main toxic metals (Cd, Hg, Pb and As) are also named sulphydryl-reactive metals. They are particularly insidious and can affect a vast array of molecular processes. Apart from, affecting essential elements status, mechanisms by which the sulphydryl-reactive metals elicit their toxic effects include: (a) Disruption of the structure and biological functions of many proteins due to high affinity for their free sulphydryl groups; (b) Challenge of pro-oxidative effects, which are compounded by the fact that the metals also inhibit antioxidative enzymes and deplete intracellular glutathione; (c) In some cases, promotion of hydrogen peroxide formation and enhancement of subsequent Fe- and Cu-induced production of lipid peroxides and the highly reactive hydroxyl radicals.

Numerous literature data substantiate the abovementioned suppositions. Miller et al. (15) showed that

Hg^{2+} acts pro-oxidatively reacting with superoxide, and provided evidence for catalytic dismutation of superoxide by this metal. Part of the irreversible loss of glutathione is due to the Hg-provoked inhibition of glutathione reductase, the enzyme used to recycle oxidized glutathione, and return it to the pool of available antioxidants (14). At the same time, Hg also inhibits glutathione synthetase, impairing the synthesis of new glutathione. Several studies reported alterations in antioxidant enzyme activities, including superoxide dismutase, catalase, glutathione peroxidase, and changes in the concentration of glutathione in Pb-exposed animals and workers (16, 17).

One aspect of our research has been based on investigation of toxic effects of sulphydryl-reactive metals (Cd and Hg) on glucocorticoid receptor (GR) structure and function. Results obtained along these lines show that reduced ability of GR to bind hormone in rat tissues in the presence of different doses of Cd or Hg can be reversed with dithiothreitol, and suggesting that toxic metals realise their effects through interaction with sulphydryl groups of GR (18, 19). Also, electrophoretical analyses of liver cytosol proteins of rats injected with 3 mg Cd/kg b.w. have shown that the examined metal possesses ability to modify GR potential to form intra- and inter-molecular disulfide bonds in both nonreducing and oxidizing (presence of hydrogen peroxide) environments. These results suggested that Cd affected the redox state of the receptor, possibly by binding to its sulphydryl groups (20).

The majority of the above mentioned effects of metals are essentially based on their influence on gene expression either through direct interaction with metal responsive transcription factors, or indirect influence on the function of signal transduction molecule (21). Among a variety of literature data considering effects of metals on gene expression (21–23) are our results, which have shown that Cd alters physiological response of rat liver cells to glucocorticoids by interfering with the GR-dependent transcriptional regulation of tyrosine aminotransferase gene (24, 25).

Cellular stress response to metals

Procaryotic and eukaryotic cells respond to physical and chemical stress by increasing the transcription of specific genes that encode the class of proteins termed heat stress proteins (Hsp). This response is believed to represent a transient reprogramming of gene expression and biological activity, which serves to protect sensitive cellular components from irreversible damage, and assists in the rapid recovery after the stress is removed or ceases. Originally termed heat shock response and heat shock proteins because of the induction of these proteins following hyperthermia, the signalling mechanism involved in its initiation is sensitive to variety of physical and chemical insults, including metals (26). That is why in this review, cellular response to stress will be called stress response, and Hsps will be considered as a subset of stress proteins, which include all proteins induced under stress conditions. The synthesis of stress proteins can be induced under a variety of different stress conditions, including elevated level of metals in the organism. Although, understanding of the relationships between metals and their capacity to induce stress response is incomplete, these relationships are important because they may reveal information regarding mechanisms of toxicity, cellular defence mechanisms against metal toxicity, and biochemical responses which can be exploited as biomarkers of exposure and toxicity of metals. This review will be focused on the role of two main classes of stress proteins in defence mechanisms. These are MTs and Hsps, proteins that are usually induced in cellular response to stress provoked by metals.

Metallothioneins

In the adaptive cell response to metal toxicity, the first defence line is attributed to MT. Defence against Cd toxicity, as a function of MT, was recognized during the initial characterization, and gained greater credence following the discovery that MT is induced by Cd and accumulates in tissues (27). A large number of studies on metal-dependent induction of MT, metal sequestering, metal-induced resistance to toxicity, and evolution of metal tolerance now support the general concept that MT is involved in protective responses to metal exposure (28).

MTs comprise a family of low molecular weight, ubiquitous and evolutionary conserved intracellular proteins that serve as a storage depot for Cu and Zn, and scavenger of sulphydryl-reactive metals that enter the cell (29). MTs are nonenzymatic proteins of 6–7 kDa, ubiquitous in the animal kingdom, and have an unusual primary structure with 30% of cysteine residues (30). Because of their highly flexible structure and high content of thiol groups, MTs can accommodate the binding of many metals with different order of affinity in two metal-thiolate clusters, reflecting the domain structure of protein (30). Hg and Cd have higher affinity for thiol groups of MT than Zn (31). Each molecule of MT binds 7 bivalent cations, such as Zn and Cd, or 12 monovalent ones, such as Cu(I). Bivalent metal cations are bound by four tetrahedrally arranged thiolate bonds (30). Binding of metal ions is not static, and the metal-MT complex is characterized by high kinetic reactivity, which confers the ability to participate in rapid intra- and inter-molecular metal transfer and exchange reactions (32). Metal ions bound to MT are blocked, and interaction with free sulphydryl groups of other proteins is disabled. At the same time, Zn pressed out from MTs or from other Zn-proteins by another metal ion, forms complexes with metal-inducible transcription factors (MTFs) and activate them to interact with the metal regulatory elements (MREs) in the promoter region of *mt* gene, turning on the syn-

thesis of MT protein (33). Induction of MT provides increased binding capacity for both toxic metals (protective role) and Zn (physiological role). Postulated mechanism underlying MT protective ability is presented in *Figure 1*.

Importance of MT in the protection against toxic metals is substantiated by numerous data (27–29, 33, 34). Mammalian cell lines having more *mt* gene copies and higher level of MT, survive exposure to Cd in culture media (35). MT-null mice, genetically engineered to have inactivated *mt* genes, died within three days of exposure to Cd from drinking water, while untreated one did not exhibit any signs of Cd toxicity (36). Cell lines that fail to produce MT due to gene hypermethylation are extremely sensitive to Cd poisoning (37). Related studies have also shown that MT may protect various cell types against poisoning by Hg, Cu and Bi, although less efficiently than against Cd (38). The MT detoxifying function was also confirmed *in vivo* by the investigations performed on testicles as the organ most sensitive to Cd poisoning (39). Our studies on *in vivo* modifications of the GR (18, 24, 40) approved the physiological role of MT in Cd detoxification by revealing an inverse correlation between the magnitude of Cd effects on GR binding capacity and MT concentration (24, 40–42).

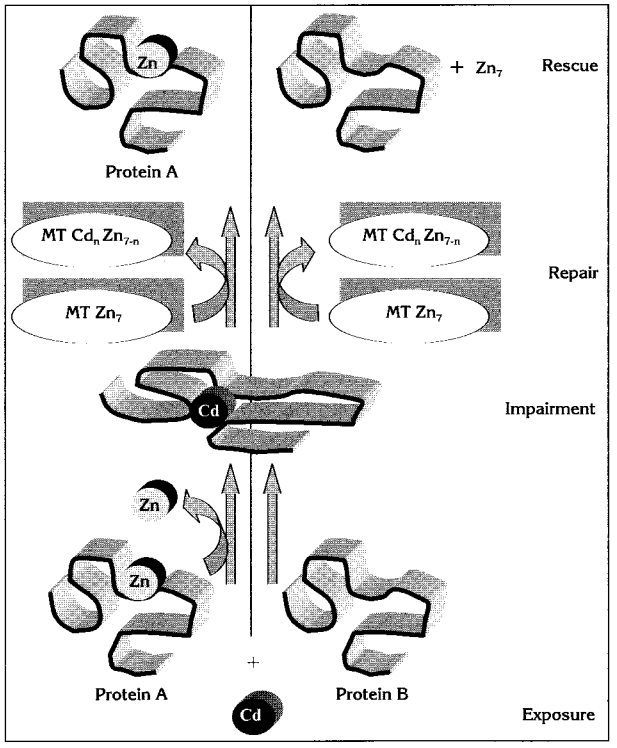

Figure 1. Schematic model for MT-based detoxification mechanism. Exposure to Cd results in Zn displacement (Protein A) or in direct binding to protein (Protein B). This results in structural alterations and functional impairment of the Cd-protein complex. Cd-Zn exchange or abstraction of Cd mediated by Zn-MT results in restoration of structure and functional state of proteins A and B.

Heat shock proteins

When the level of metal ions in the cell overcomes the capacity of MT, as a first defence line, to bind and detoxify them, the concentration of free metal ions considerably increases, and eventually causes misfolding, aggregation or denaturation of proteins through provoking oxidative stress and interacting with protein thiols (26). Appearance of damaged proteins in the cell serves as signal that triggers the activation of *hsp* genes, the products of which, playing the role of molecular chaperones, could prevent and repair cellular damage (43, 44). However, since human *hsp*70 promoter was shown to harbour metal regulatory elements (45), the induction of Hsps by metal ions might be triggered not only by the accumulation of aberrant proteins, but also by direct action through MTF. How do eukariotyc cells sense a presence of stress signal? It is commonly held that the stressful signals, including metals, provokes accumulation of non-native proteins. This, in turn, results in the cellular requirement for molecular chaperones, especially Hsp70 and Hsp90, which serve to repair denaturated and aggregated misfolded protein (*Figure 2*). Under normal conditions, Hsp70 is in the complex with HSF1 monomer. Increased level of damaged proteins, resulting from the action of a variety of stress-provoking factors, sequesters Hsp70 from complex with HSF1, since Hsp70 have high affinity for bared hydrophobic regions of unfolded proteins. As a consequence of Hsp70-damaged proteins interactions, DNA-binding activity of HSF1 is derepressed and HSF1 is activated by trimerization and phosphorylation. Activated HSF1 translocates to the nucleus and binds to specific DNA sequences located in the promoter regions of *hsp* genes (HSE), resulting in stress-induced transcription (46). As the synthesis of Hsps increases to the levels proportional to the level of aberrant proteins, Hsp70 relocalize to the nucleus and binds to the HSF1 transcriptional transactivation domain, thereby repressing transcription of *hsp* genes (47).

Hsps are highly conserved, ubiquitous and abundant intracellular proteins, which are divided into different families according to molecular size (Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsp). In general, eukaryotes possess at least two copies of most *hsp* genes: a stress-inducible gene, and a constitutively expressed one (48). Majority of these proteins in mammalian cells exert a fundamental role of molecular chaperones, being essential for correct folding, assembly, and intracellular translocation of newly synthesized proteins (49). Under stress conditions, a common protective function of induced Hsps is to hold damaged proteins in the form of soluble folded intermediates competent for refolding, which function as kinetic traps to prevent off-pathway intermediates and formation of aggregates (50). While prolonged exposure to conditions of extreme stress is harmful and can lead to cell death, induction of Hsp synthesis

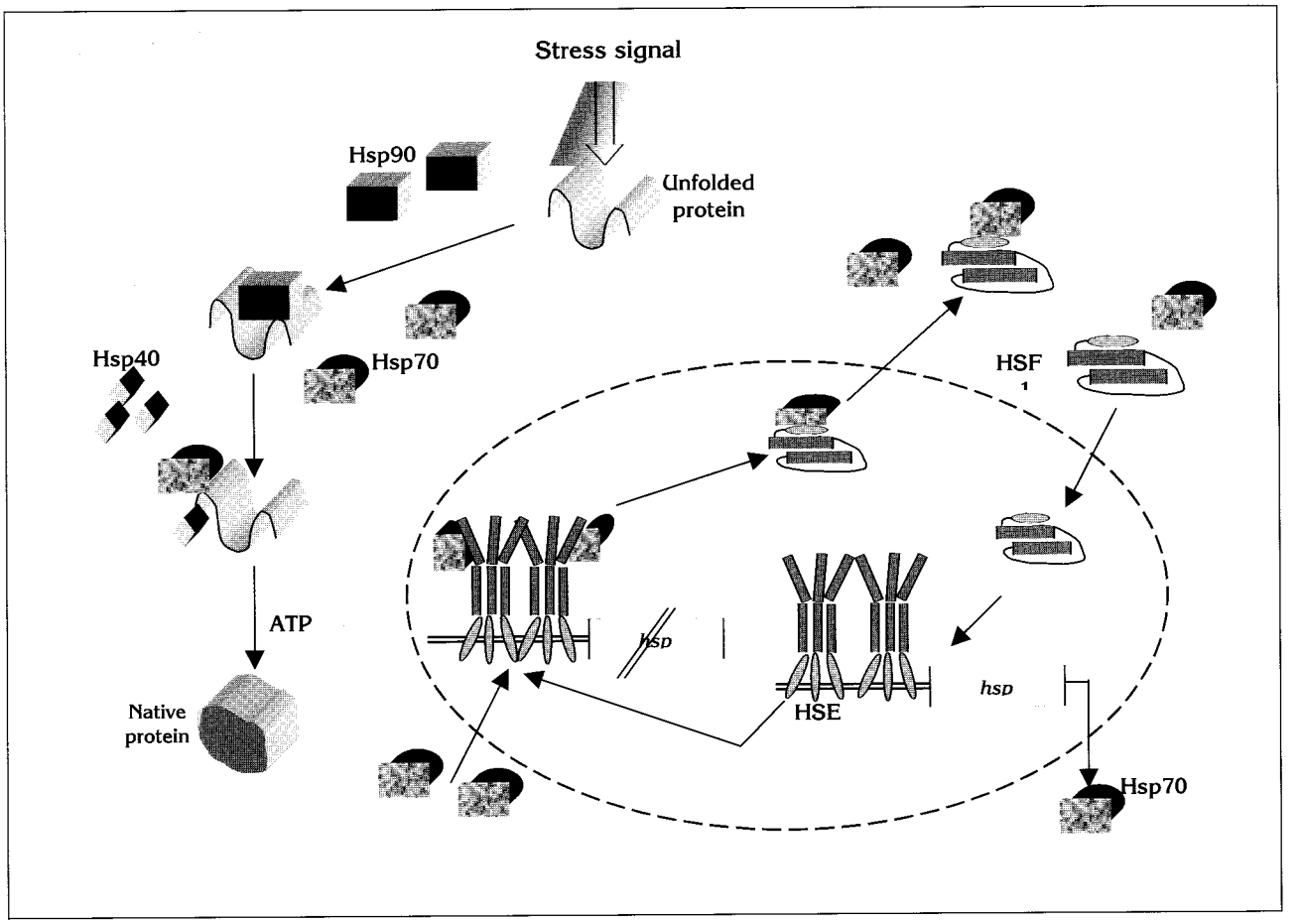

Figure 2. A possible mechanism for regulation of cellular stress response. Binding of Hsps to unfolded protein occurs under stress signal after disruption of Hsp70-HSF1 heterocomplex. As a consequence of the release of interacting chaperones, HSF1 monomers translocate to the nucleus, oligomerize to a trimeric state, become phosphorylated, and bind to HSE (heat shock elements). As a consequence, transcription of hsp genes and the synthesis of Hsps increase. When the stressful signal ceases, Hsp70 relocalizes to the nucleus and binds to HSF1, thereby repressing hsp gene transcription, which leads to dissociation of trimers and refolding of HSF1to the inert monomeric state.

can result in stress tolerance and cytoprotection against stress-induced molecular damage (51).

The enhanced synthesis of Hsps by metals can vary considerably, and is influenced by factors such as specific metal tested, the chemical form of the metal, dose and duration of exposure, tissue or cell type, and developmental stage and age of an organism. A large body of evidence confirmed that stress-induced Hsps have a protective role against metal-provoked toxicity. The data of Goering et al. (52) demonstrated that Cd induces elevation in *de novo* expression of *hsp70* in rat liver, and that this synthesis occurred prior to overt hepatic injury. They also showed that Hg(II) induces synthesis of Hsps in rat kidney prior to detection of nephrotoxicity by classical biochemical and functional assays (53). These metal-induced changes in Hsps gene expression appeared to be target organ specific. Further, Bauman et al. (54) have shown that incubation of rat hepatocytes with Cd, Hg, Ni, As and Zn results in the induction of Hsp70, Hsp90 and MT, and that the intensity of this induction depends on metal species. The existence of metal-specific enhancement of Hsp induction by low doses of metals in conditions of self- and cross-sensitisation was also noticed (55). The results of studies performed in our laboratory confirmed the capability of Cd to enhance the levels of MT, Hsp70 and Hsp90 in the liver cytosol of rats injected with different doses of the metal (*Figure 3*) (41, 42, 56). The results also suggested that Cd-induced reduction of the GR binding capacity seen *in vitro* was prevented in intact animals by the elevated level of Hsp90 within the GR heterocomplexes (56).

One more Hsp, Hsp32, is known to be induced by heat and various metal ions probably as a consequence of provoked oxidative stress (57–60). This protein is identified as inducible isoform of heme oxige-

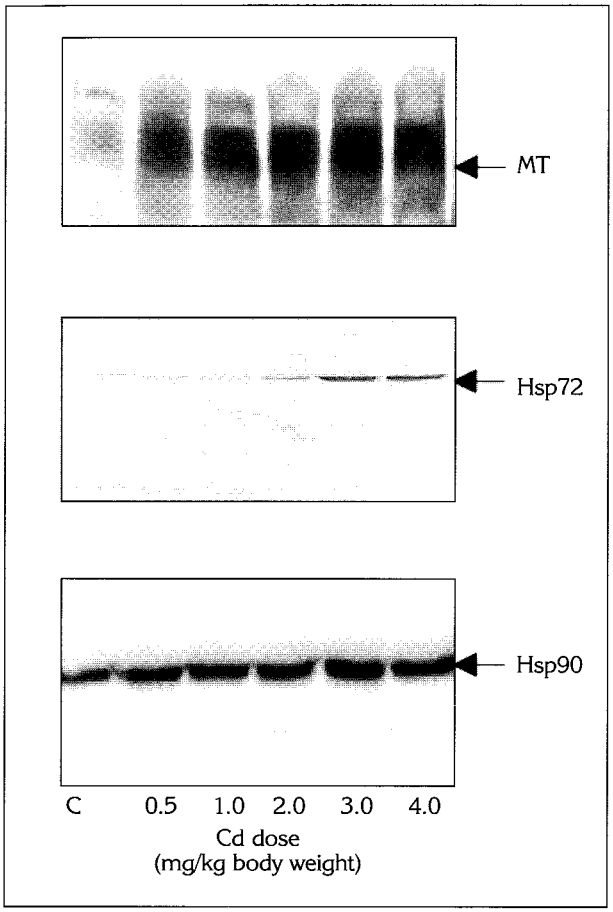

Figure 3. MT, Hsp70 and Hsp90 induction in the liver cytosol of rats administered with different Cd doses. Carboxymethylated MT from heat-stable fraction of rat liver cytosol was resolved by low molecular weight SDS-PAGE and visualised by sensitive silver staining procedure. In order to detect Hsps, cytosol proteins were electrophoretically separated and transferred to nitrocellulose membranes. Hsp72 was detected using monoclonal antibody against inducible form of Hsp70, while Hsp90 was detected with AC88 monoclonal antibody.

nase (HO1), an enzyme included in the process of heme degradation (61). It cleaves the heme tetrapyrrole ring structure to biliverdin, which is subsequently converted to bilirubin *via* action of biliverdin reductase. It has been postulated that the induction of HO1 enhances cellular resistance to oxidative injury, because bilirubin and biliverdin have the ability to suppress oxidative stress (62)

Cellular stress response is a complex fundamental biological phenomenon that includes activation of superfamily of genes (»stress genes«), in case of necessity, and dependently on the type of stress. All or some of these genes and their products may have to function in harmony with one another, and cope with cellular needs to survive. Under metal intoxication, cell activates expression of at least two families of genes, MTs and Hsps, which both confer adaptation or tolerance to variety of metal-provoked toxic effects. More understanding about cellular defence mechanisms would help in keeping our physiological systems vigilant and our body healthy, fighting out the stress-related events effectively.

Acknowledgement: This work was supported by the Ministry of Science, Technology and Development of the Republic of Serbia, Serbia and Montenegro, project #1654. The author is grateful to Dr. Gordana Matić for critical reading of the manuscript.

ĆELIJSKI ODGOVOR NA STRES – ODBRANA OD TOKSIČNIH EFEKATA METALA

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Kratak sadržaj: Sve žive ćelije odgovaraju na različite tipove stresa povećavanjem transkripcije specifičnih gena koji kodiraju klasu proteina nazvanih stres proteini. Taj odgovor predstavlja prolazno reprogramiranje ekspresije gena i biološke aktivnosti, i služi da zaštiti osetljive ćelijske komponente od oštećenja, i pomogne u brzom oporavku posle uklanjanja ili prestanka delovanja stresa. Sinteza proteina stresa može biti indukovana pod delovanjem različitih stresogenih uslova, uključujući i povećani nivo metala. Pošto je razumevanje odnosa između metala i njihovog kapaciteta da indukuju odgovor na stres nedovoljno poznato, njihovo proučavanje je važno zato što može dati informacije o mehanizmima toksičnosti metala, ćelijskim odbrambenim mehanizmima i biohemijskim odgovorima koji se mogu koristiti kao biomarkeri izlaganja ili toksičnosti metala. Ovaj revijski članak je fokusiran na dve klase stres proteina, metalotioneina (MT) i proteina toplotnog stresa (Hsp), koje se najčešće indukuju u odgovoru na stres provociran metalima. Sumirani su rezultati istraživanja toksičnih efekata metala i njihove sposobnosti da indukuju ćelijski odgovor na stres.

Ključne reči: metali, ćelijski odgovor na stres, metalotioneini (MT), proteini toplotnog stresa (Hsp)

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Received: October 18, 2003

Accepted: December 5, 2003