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A POSSIBLE ROLE OF METALLOTHIONEIN AND HEAT SHOCK PROTEINS IN GLUCOCORTICOID RECEPTOR PROTECTION AGAINST CADMIUM INTOXICATION

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Abstract - The participation of metallothionein (Mt) and heat shock proteins (Hsps) in protection of glucocorticoid receptor (GR) binding capacity from a toxic metal cadmium (Cd), was investigated. Specific binding of a glucocorticoid to GR in the rat liver cytosol was studied after *in vitro* and *in vivo* Cd treatment. Reduction of the GR binding capacity observed after *in vitro* treatment was proportional to the applied metal concentrations. In animals administered different Cd doses, GR binding capacity was not reduced, except in those that received the highest dose, and a concomitant elevation of Mt, Hsp70 and Hsp90 levels was detected. The results led to the assumption that Cd-induced reduction of the GR binding capacity noticed *in vitro,* was prevented in intact animals by the elevated levels of Mt and Hsps.

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INTRODUCTION

Cadmium (Cd) is a nonessential heavy metal accountable for many toxic effects in various living organisms, including man (G o e r i n g *et al.* 1995). Although the molecular mechanisms responsible for Cd toxicity are not well understood, it is believed that its major toxic effects are the consequence of either its high affinity for protein thiol groups or the competition with essential metal ions (V a l l e e and U l m e r 1972; S t a c e y 1986).

Cd triggers a complex reprogramming of the expression of both heat shock protein (Hsp) and metallothionein (Mt) genes, that results in increased synthesis of the corresponding proteins (B a u m a n *et. al.* 1993; G o e r i n g *et al.* 1993; S t e i n e r *et al.* 1998; W i e g a n t *et al.* 1998). Mt, as a protein with 20 cysteine residues, is the first defence mechanism in the process of Cd detoxification (A n d r e w s 1990). Only when all Mt thiol groups are occupied with Cd, the metal ions attack thiol groups of other proteins, generating an array of abnormal or denatured proteins. The appearance of such proteins in the cell represents a signal for the induction of Hsps (N o v e r 1991). Serving as chaperones, Hsps ensure protection of the cell constituents under unfavourable conditions, including heavy metals intoxication (G o e r i n g and F i s h e r 1995). Among numerous regulatory proteins chaperoned by Hsps are glucocorticoid receptor (GR) and other steroid hormone receptors which associate with Hsp90, Hsp70 and Hsp56 forming the untransformed heterooligomeric complexes (P r a t t and T o f t 1997).

S i m o n s *et al.* (1990) reported that Cd applied *in vitro* affected multiple functions of the GR from hepatoma tissue culture cells, presumably by binding to vicinal thiols in the steroid binding domain of the receptor. Our previous studies (D u n d j e r s k i *et al.* 1992, 1996) demonstrated that Cd reduced the GR binding capacity in the rat liver cytosol and also suggested the interaction of the metal with thiol groups of the receptor.

Based on the fact that GR is associated with Hsps and that Cd activates Mt-dependent defence mechanisms in the cell, the main goal of the present study was to examine the participation of Mt and Hsps in GR protection against Cd-induced damage. The results presented in this paper show a correlation between the GR protection and the extent of Mt and Hsps induction by Cd in the rat liver.

MATERIAL AND METHODS

Chemicals

[1,2,4(n)-^{3}H] Triamcinolone acetonide (TA, specific activity 740 Gbq/mmol) and ^{125}I-labelled sheep antimouse IgG were obtained from Amersham International, Amersham, UK. Monoclonal antibodies N27F3-4 (anti-Hsp72/Hsp73), C92F3A-5 (anti-Hsp72) and AC88 (anti-Hsp90) were the products of StressGen (Victoria, British Columbia, Canada). Unlabelled TA and rabbit Mt were purchased from Sigma (St. Louis, MO, USA).

Animals

Male rats of Wistar strain (2-2.5 months old; 200 250 g b.w.) were reared under standard laboratory conditions with 12:12 *h* light/dark cycle, at 22 °C. Cadmium chloride ($CdCl_2$) dissolved in 0.9% $NaCl_2$ was administered *i.p.* in the doses of 0.5 4 *mg* Cd/kg b.w., 16 *h* before death. Control rats received 0.9% NaCl by the same schedule.

Preparation of the liver cytosol

The livers were perfused *in situ* with cold saline and homogenates were prepared from at least three animals *per* group, in 2 vol. (w/v) of 50 mM Tris buffer, pH 7.55 containing 0.25 M sucrose, 25 mM KCl and 10 mM $MgCl_2$. After centrifugation (10 min, 6000 g, 4 °C and 1 *h*, 105 000 g 4 °C), the upper lipid layers were aspirated and cytosols stored in liquid nitrogen until use.

Hormone binding studies

The GR binding capacity was determined by incubation (18 *h*, 0 °C) of triplicate 0.1 mL cytosol aliquots with 100 nM [^{3}H]TA dried under the nitrogen stream. Nonspecific binding was evaluated from the parallel incubations in 100- fold molar excess of radioinert TA. The unbound steroid was removed by a dextran-charcoal competitive binding technique. Specific binding, expressed in pmol bound hormone *per* mg protein, was taken as the difference between the radioactivity measured in the absence of unlabelled TA (total binding) and in its presence (nonspecific binding).

In *in vitro* experiments, GR binding capacity was measured by the same procedure, after the incubation (3 *h*, 0 °C) of control cytosols with different Cd concentrations (2 -16 μg Cd/mL).

Metallothionein determination

Heat stable fraction of liver cytosol was carboxymethylated for 15 min at 50 °C with 1 M iodoacetic acid (O t s u k a *et al.* 1988), boiled in ^{2X}SDS-sample buffer and subjected to SDS-PAGE. Carboxymethylated proteins were resolved according to L a e m m l i's (1970) procedure modified for separation of low molecular weight polypeptides. The samples were loaded on 1 mm thick, 19.5% acrylmide 0.5% bisacrylamide slab gels prepared in SDS-Tris buffer pH 9.3. After electrophoresis (3 *h*, 135 V, 4 °C), the gels were subjected to combined Coomassie blue-silver staining procedure (D e M o r e n o *et al.* 1985), scanned and analysed by the ImageQuant computer program. Purified rabbit Mt was used as a marker protein.

SDS-PAGE

Proteins were resolved according to L a e m m l i (1970) in 10% SDS-polyacrylamide gels using Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA). Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) were simultaneously run as molecular mass references.

Immunoblotting

Western transfer of proteins from acrylamide gels to nitrocellulose membranes was performed in 25 mM Tris buffer, pH 8.3 containing 192 mM glycine and 20% (v/v) methanol, at 135 mA overnight in Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Unbound sites on the membranes were blocked by 20 mM Tris buffer pH 7.4 containing 150 mM NaCl, 0.125% BSA and 0.1% Tween 20. The membranes were incubated by rocking (16 *h*, 4 ° C) in the blocking buffer with 1 μg/mL N27F3-4, 1 μg/mL C92F3A-5, or 3.2 μg/mL AC88 monoclonal antibody, for detection of the Hsp72/73, Hsp72 or Hsp90, respectively. The immunoblots were then incubated with the ^{125}I-labelled counter-antibody. The membranes were exposed to PhosphorImager screen, scanned in PhosphorImager (Molecular Dynamics), and analysed by the ImageQuant computer program.

Miscellaneous

Total Cd content in liver cytosols was determined by digesting cytosol aliquots in 1 M HNO_3 for 1 *h* and centrifuging (15 min, 1500 rpm), followed by atomic absorption spectrometry (Perkin Elmer 4000).

Protein content in the cytosols was determined by the method of L o w r y *et al.* (1951) with bovine serum albumin as a standard.

RESULTS

In order to examine *in vitro* Cd effects on GR binding capacity, rat liver cytosol was incubated with metal concentrations ranging from 2 to 16 μg Cd/mL cytosol. These concentrations were selected such as to be comparable with those determined by atomic absorption spectrophotometry in the cytosols after *in vivo* administration of different Cd doses (Table 1).

Table 1. Concentration of Cd in the liver cytosol of rats injected with different Cd doses.

Cd dose (*mg/kg* b.w.)	Cd concentration* (μg/mL cytosol)
Control	0.00
0.5	1.92 ± 0.31
1	4.89 ± 0.26
2	7.31 ± 0.18
3	9.97 ± 0.48
4	12.78 ± 0.38

*The values represent the means ±SE from four independent determinations of Cd concentration by atomic absorption spectrometry.

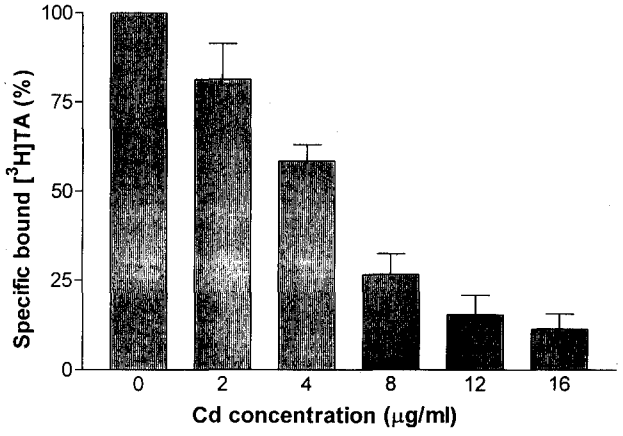

Fig. 1. *In vitro* effect of Cd on steroid-binding capacity of GR in the rat liver cytosol. Liver cytosol was preincubated for 3 *h* at 0 °C with indicated Cd concentrations. Specific binding of the hormone was measured by incubating the cytosol aliquots (18 *h*, 0 °C) with 100 nM [^{3}H]TA± 50 μMTA and removing the free steroid by dextran-charcoal. The specific [^{3}H]TA binding was expressed as a percentage of that measured in the untreated cytosol. The values represent the mean ±SE from four independent experiments done in triplicates.

It was found that applied Cd concentrations led to a reduction of GR ability to bind TA in a dose-dependent manner (Fig. 1). The GR binding capacity was diminished by the highest Cd concentration (16 μg/mL) by nearly 90% in comparison with that in untreated cytosol.

When GR binding capacity was assayed 16 *h* after *i.p.* administration of different Cd doses (0.5-4 *mg/kg* b.w.) to intact animals, it was noticed that GR ability to bind the hormone remained unchanged except when the highest Cd dose (4 *mg/kg* b.w.) was applied when a slight reduction was observed (Fig. 2).

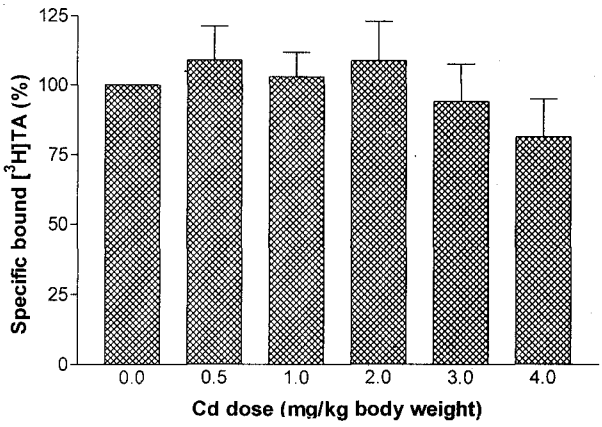

Fig. 2. Steroid-binding capacity of the GR in the rat liver cytosol after administration of indicated Cd doses. Specific binding of hormone was measured by incubating the cytosol aliquots (18 *h*, 0 °C) with 100 nM [^{3}H]TA ±50 M TA and removing the free steroid by dextran-charcoal. The specific [^{3}H]TA binding was expressed as a percentage of that measured in the untreated cytosol. The values represent the mean ±SE from four independent experiments done in triplicates.

The lack of the reduction of GR binding capacity after *in vivo* Cd treatment, as opposed to the strong reduction noticed after *in vitro* application of the metal, prompted us to examine the induction of Mt, Hsp70 and Hsp90, as possible participants of defence mechanism(s) activated in response to Cd intoxication. The results displayed in Fig. 3 indicate that the applied Cd doses induced Mt synthesis in the rat liver. The maximal extent of Mt induction was noticed upon administration of 2 *mg* Cd/*kg* b.w. when the level of the protein was approximately 2.7 fold higher than that in untreated animals. A decline of Mt induction, detected after injection of 3 and 4 *mg* Cd/*kg* b.w., could be explained by cytotoxic effects of these doses, diminishing the normal detoxifying mechanisms.

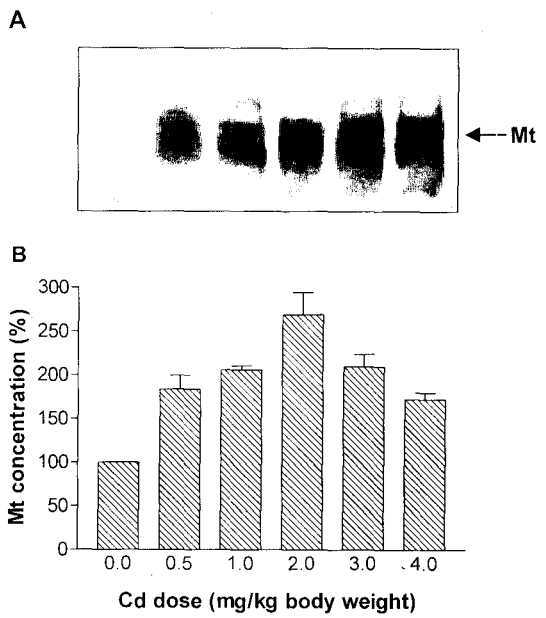

Fig. 3. Mt induction in the liver cytosol of rats administered different Cd doses. (A) Carboxymethylated Mt from heat-stable fraction of rat liver cytosol was resolved by low molecular weight SDS-PAGE in 19.5% acrylamide-0.5% bis-acrylamide gels and visualised by a sensitive Coomassie blue-silver staining procedure. The representative electrophoretogram is shown. The arrow indicates the position of rat liver Mt. Lane 1: Mt from cytosol of control rats; Lanes 2-6: Mt from liver cytosols of rats administered Cd doses of 0.5, 1, 2, 3 and 4 *mg/kg* b.w., respectively. (B) Data obtained after laser scanning and quantification of Mt bands by ImageQuant software. Relative Mt concentration is expressed in relation to the untreated control taken as 100%. The values represent the mean ±SE from three independent experiments.

Since the increased amounts of Hsp70 and Hsp90 during cellular stress response might confer protection of the GR structure and function, their cytosolic levels upon Cd treatment were determined. Immunoblot detection with two different anti-Hsp70 monoclonal antibodies (N27F3 - 4 recognising both constitutive-Hsp73 and inducibleHsp72 forms of the protein and C92F3A-5 directed against Hsp72) was performed (Fig. 4). Quantitative analysis by PhosphorImaging provided numerical data presented in Fig. 4C. When the level of total Hsp70 was determined, a modest Cd-related increase (up to 30%) was noticed (Figs. 4A and 4C open bars). However, when the antibody recognising only Hsp72 was used, it was found that Cd doses of 1, 2, 3 and 4 *mg/kg* b.w. led to 1.5-, 2-, 7.7- and 5- fold elevation over the basal level, respectively (Figs. 4 B and 4 C-hatched bars).

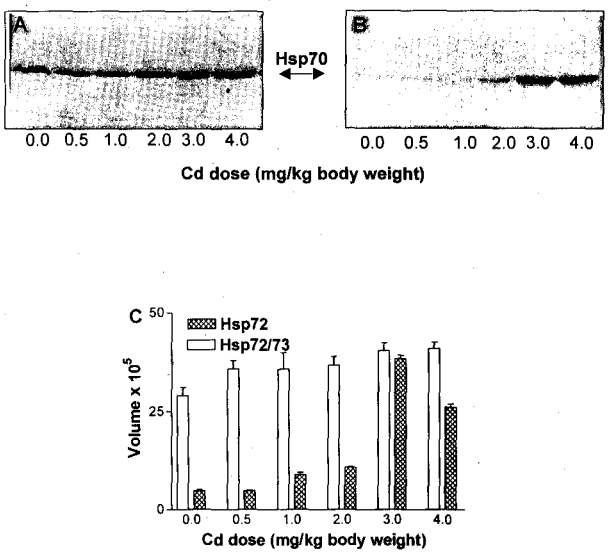

Fig. 4. Hsp70 in the liver cytosol of rats administered different Cd doses. Cytosol proteins (40 μg) from rats administered different Cd doses were separated by 10% SDS-PAGE, and transferred to nitrocellulose membrane. Hsp70 was detected using N27F3-4 (A) or C92F3A-5 (B) monoclonal antibody, followed by ^{125}I-labelled counter antibody. The representative scan obtained by Phosphor-Imaging is shown. The arrow indicates the position of Hsp70. Lane 1 - Hsp70 from cytosol of control rats; Lanes 2-6 - Hsp70 from the liver cytosols of rats administered Cd doses of 0.5, 1, 2, 3 and 4 *mg/kg* b.w., respectively. (C) Data obtained after quantification of immunoreactive bands by ImageQuant software. The values represent the mean ±SE from three independent experiments.

Immunoblot determination of Hsp90 in the liver cytosol of untreated and Cd-treated animals using AC88 monoclonal antibody, showed that the doses of 1-3 *mg/kg* b.w. caused a dose-dependent increase in Hsp90 concentration, as well. The elevation of Hsp90 level in the liver cytosol ranged from 50-110% in comparison with the untreated cytosol (Fig. 5).

DISCUSSION

In the present study we examined the correlation between Cd effects on GR binding capacity and on the levels of Mt and Hsps in the rat liver cytosol. The reduction of the hormone binding to the receptor observed in *in vitro* experiments was proportional to the supplemented Cd concentrations. The absence of such an effect after administration of increasing Cd doses, showed that the metal-treated animals probably activa-

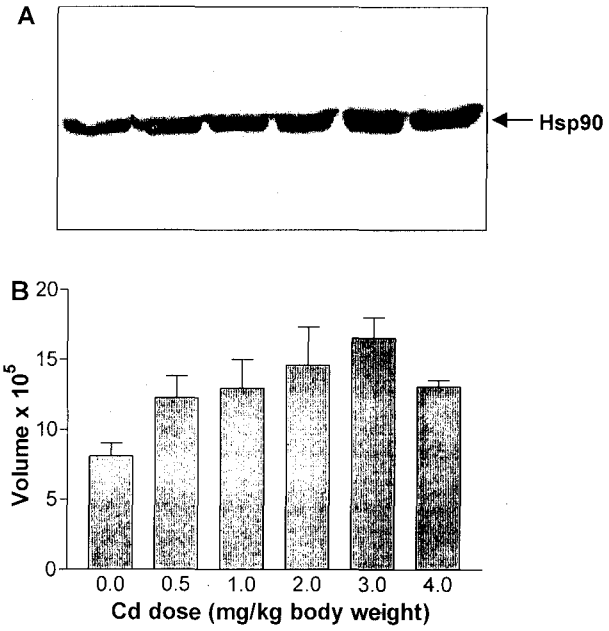

Fig. 5. Hsp90 in the liver cytosol of rats administered different Cd doses. (A) Cytosol proteins (40 μg) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. Hsp90 was detected with AC88 monoclonal antibody followed by ^{125}I-labelled counter antibody. The representative scan obtained by PhosphorImaging is shown. The arrow indicates the position of Hsp90. Lane 1: Hsp90 from the cytosol of control rats; Lanes 2-6: Hsp90 from the liver cytosols of the rats administered with Cd doses of 0.5, 1, 2, 3 and 4 *mg/kg* b.w., respectively. (B) Data obtained after quantification of immunoreactive bands by Image-Quant software. The values represent the mean ±SE from three independent experiments.

ted some defence mechanism(s) which ensured protection from the toxic effects. The first candidates possibly responsible for the GR protection could be Mt and Hsps.

Mt, as a metal-inducible protein, represents the first line of defence in cellular response to Cd intoxication owing to its high cysteine content and marked Cd affinity for protein thiol groups (A n d r e w s 1990; K a g i 1991). In the present study, doses up to 2 *mg* Cd/*kg* b.w. proved to be increasingly efficient in all Mt, Hsp70 and Hsp90 induction. These proteins present in elevated levels were probably sufficient to protect GR binding capacity from the reduction by the metal. Cd dose of 3 *mg/kg* b.w. was less effective than the lower one (2 *mg/kg* b.w.) in stimulating Mt synthesis, but was the most efficient in Hsps induction. It is likely that at this Cd dose, Hsps prevailed over Mt in GR protection. Namely, under conditions of relatively high Cd and low Mt concentration, free Cd ions might increase and cause misfolding, aggregation or denaturation of proteins, including GR, through the interaction with their thiols (N o v e r 1991; J u n g m a n n *et al.* 1993). Appearance of damaged proteins in the cell after Cd treatment, could serve as a signal that triggers the activation of *hsp* genes, the products of which, playing the role of molecular chaperones, could prevent and repair cellular damage (H i g h t o w e r *et al.* 1994). Regulation of Hsps synthesis is supposed to involve binding of Hsp70 to damaged proteins, releasing the heat shock transcription factor (HSF) from Hsp70-HSF complexes, and activation of *hsp* genes transcription (M o r i m o t o *et al.* 1996). However, since human *hsp70* promoter was shown to harbour metal regulatory elements (W u *et al.* 1986), the induction of Hsps by Cd might be triggered not only by the accumulation of aberrant proteins, but also by direct action of the metal-inducible transcription factor(s).

The defending mechanisms, however, failed to protect GR hormone-binding activity in the liver cytosol upon administration of Cd dose of 4 *mg/kg* b.w.. The reduction of GR binding capacity provoked by this dose coincided with a decline in the rate of all three examined proteins synthesis. This observation could be explained by possible cytotoxic effects of this relatively high metal dose that could diminish or even prevent normal detoxifying mechanisms.

It was previously established that some Hsps, including Hsp90 and Hsp70 are constituents of untransformed GR heterocomplexes. Intensive studies on their role in GR functioning have clearly shown that Hsp90 is required for maintaining the receptor hormone-binding domain in a proper conformation, as well as for transactivation activity and intracellular shuttling of the receptor (P r a t t and T o f t 1997; Č v o r o *et al.* 1998). Therefore, it was reasonable to expect that Cd-induced elevation of Hsp90 level might stimulate the association of this protein with GR and thus, the protection of the receptor binding activity. As for Hsp70, however, its chaperoning function in the cell is well documented, but its role within GR heterocomplexes is still a matter of debate. Thus, further investigations are necessary in order to elucidate its participation in GR heterocomplexes, as well as to evaluate a particular contribution of Hsp90 and Hsp70 to the GR protection from Cd-induced damage.

It is evident from the data presented herein that Cd elicits heat shock response characterised by elevated synthesis of Hsps in the rat liver. The results also confirm previous observations that the induction of Mt, as a typical cellular response to this metal, represents an early detoxifying process the climax of which gets reac-

hed earlier than that of heat shock proteins induction (B a u m a n *et al.* 1993; O v e l g o n n e *et al.* 1995).

Based on previous studies, it was already proposed that functional modifications of GR represent a part of Cd toxicity (D u n d j e r s k i *et al.* 1992, 1996). Our results suggest that increased levels of Mt, Hsp70 and Hsp90 could be responsible for GR protection against Cd insult and thus represent an example of molecular mechanisms comprising Cd detoxification. Further studies along these lines will be focused on GR-associated Hsps and possible alterations in the untransformed receptor heterocomplexes composition after Cd intoxication.

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МОГУЋА УЛОГА МЕТАЛОТИОНЕИНА И ПРОТЕИНА ТОПЛОТНОГ СТРЕСА У ЗАШТИТИ ГЛУКОКОКОРТИКОИДНОГ РЕЦЕПТОРА У УСЛОВИМА ТРОВАЊА КАДМИЈУМОМ

ЈАДРАНКА ДУЊЂЕРСКИ, ЈЕЛЕНА ПРЕДИЋ, ТАЊА КОВАЧ, НАДА ПАВКОВИЋ, ЉУБИЦА ИВАНИШЕВИЋ, АЛЕКСАНДРА ЧВОРО и ГОРДАНА МАТИЋ

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Испитивано је учешће металотионеина (Mt) и протеина топлотног стреса (Hsp) у заштити глукокортикоидног рецептора (GR) од токсичног деловања кадмијума (Cd). Специфично везивање глукокортикоида за GR у цитосолу јетре пацова праћено је после *in vitro* и *in vivo* третмана кадмијумом. Запажено је да је у *in vitro* условима смањење способности GR да везује хормон пропорционално примењеној концентрацији метала. Код животиња инјецираних различитим дозама Cd, капацитет GR за везивање хормона је непромењен осим у присуству највеће дозе, и истовремено се уочава повећање концентрације Mt, Hsp70 и Hsp90. Добијени резултати указују да је смањена способност GR за везивање хормона запажена након *in vitro* третмана кадмијумом, код животиња инјецираних кадмијумом спречена повећаном индукцијом Mt и Hsp.