Effects of cadmium ions on the initial stage of translation and the cell death in mouse liver

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Key words: cadmium, tRNA, leucyl-tRNA synthetase, apoptosis, necrosis.

Summary. Objective. To evaluate in vivo and in vitro effects of cadmium ions on the activities of mouse liver tRNA⁶⁰⁰⁰⁰ and leucyl-tRNA synthetase and on the type of liver cells death.

Material and methods. White laboratory mice were intoxicated by intraperitoneal injection of cadmium chloride solution (1.6 mg cadmium ions/1 kg of body weight). Total tRNAs were isolated by adding ethanol and isopropanol into the phenol-deproteinized supernatant of mouse liver homogenate. Post-mitochondrial fraction of the liver cells was used as a source of leucyl-tRNA synthetase. Acceptor activity of tRNA⁶⁰⁰⁰ and activity of leucyl-tRNA synthetase were measured in tRNA aminoacylation reaction with [¹⁴C]-labeled leucine as a substrate. An apoptotic cell death was assessed by the TUNEL assay using in situ cell death detection kit. DNA degradation was verified by electrophoresis.

Results. It was determined that 2–24 hours after intoxication with sublethal dose of cadmium ions the acceptor activity of mice liver tRNA⁶⁰⁰⁰ was decreased by 43–73% as compared to control. At the same time intervals, the activity of leucyl-tRNA synthetase was reduced about 20–30%. Experiments in vitro revealed that 10–20 μM concentrations of cadmium ions suppressed the activities of mice liver tRNA⁶⁰⁰⁰ and leucyl-tRNA synthetase by 40–98%. No significant difference was observed between the number of TUNEL positive apoptotic liver cells in the control mice and 24 hours after intoxication with cadmium chloride. Electrophoresis revealed extensive degradation of nuclear DNA.

Conclusions. Cadmium ions significantly reduce activities of tRNA⁶⁰⁰⁰ and leucyl-tRNA synthetase in vivo and in vitro. There is no significant difference between the number of apoptotic cells in the control liver specimens and in those after 24 hours of intoxication with cadmium chloride. In latter specimens DNA electrophoresis revealed as extensive degradation of DNA, which is characteristic to the cell necrosis.

Introduction

Heavy metal cadmium (Cd), a well-known environmental hazard, exerts a number of toxic effects in humans and animals. A decade ago, the International Agency for Research of Cancer attributed Cd to category 1 of carcinogens, which are carcinogenic to the humans (1). Tobacco smoke, food and industrial pollution are the main sources of Cd to the humans. It is recognized that an exposure to Cd can result in various pathologies including neoplasia, osteoporosis, irreversible renal tubular injury, anemia, etc. (1). A number of Cd-induced effects including deterioration of cell-cell adhesion, DNA-related processes, cell signaling and energy metabolism can imply that this metal acts on the different molecular targets in human organism. One of the targets for Cd is the system of protein synthesis or translation. There are some evidences that in translation, Cd activates particular initiation and elongation factors (2). The effect, however, is detectable only in transformed cells. Activation of translation can be a consequence of Cd-induced gene transcription, as it was determined for metallothionein
(3), heat-shock proteins (4) and glutathione (5). It is shown that effect of Cd on the protein synthesis in vivo depends on intoxication duration (6) and, probably, on dose of this metal. According to the data of in vitro study, Cd in low concentrations can activate both the rate and the level of translation but in high concentrations it inhibits those parameters (6).

As the system of translation is particularly complicated, its components can differently respond to Cd. There are evidences about inhibitory action of subtoxic amount of Cd on the components of protein synthesis in rat testes (7). However, in the above study, no attention was paid on intoxication time-dependent effects of cadmium. Thus, the effects and mechanisms of Cd action on protein synthesis are controversial and not entirely understood.

Acting on the different sites of cellular metabolism Cd can severely diminish cell viability resulting in the cell death (8). Interfering with death signal transduction pathways, Cd can induce two extreme types of the cell death – apoptosis and necrosis (9). Mechanisms that are behind Cd-induced cell death strongly depend on the type of the cell. The experiments aimed to elucidate these mechanisms have been done on the distinct types of tissues and cells. Therefore, they can be compared with great precaution.

The present study is designed to investigate the relationship between duration of Cd intoxication, protein synthesis and the mode of cell-death induced in mouse liver. We have found almost permanent decrease of activities of the components of priming stage of translation (tRNA and aminoacyl-tRNA synthetase) over 2-, 8- and 24-hour periods of Cd intoxication. The effects correlated with a change of the cell-death type from apoptosis to necrosis.

Material and methods

White laboratory mice (20–25 g) were used for the study (License of State Veterinary Service for Working with Laboratory Animals No. 0028). Intoxication with Cd was performed by injection of sublethal dose of CdCl₂ (1.6 mg Cd²⁺ per 1 kg of body mass) dissolved in physiological solution into abdominal cavity of mice. Control animals received injection of the same volume of physiological solution. The preparation procedures of both total tRNAs and post-mitochondrial supernatant (source of leucyl-tRNA synthetase) from mouse liver were described earlier (10).

Acceptor activity of tRNA^[leu] was determined in the 100 μl of reaction mixture, consist of 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM KCl, 4 mM ATP, 0.2 mM [³⁵S]-leucine, 250 μg protein of post-mitochondrial supernatant (source of leucyl-tRNA synthetase), and 50 μg total tRNAs. The reaction mixture was incubated at 37°C for 20 min. Reaction was stopped by adding of 0.2 ml ice-cold 10% trichloroacetic acid. Test tubes with reaction mixture were kept in ice bath for 20 min for formation of precipitate. The precipitate was collected on nitrocellulose filters and washed with 25–30 ml of ice-cold 5% trichloroacetic acid. Radioactivity was measured in liquid scintillation counter (Deltal 300, Tracer Europe, Netherlands), the efficiency of counting 60%. Acceptor activity of tRNA^[leu] was evaluated by the formation of [³⁵S]-leucyl-tRNA^[leu]. Activity of leucyl-tRNA synthetase was measured in post-mitochondrial supernatant by the initial rate of tRNA^[leu] aminoacylation with [³⁵S]-labeled leucine (10).

Apoptosis of liver cells was immunohistochemically detected by the TUNEL assay using in situ cell death detection kit, AP (Roche). Sections of formalin-fixed and paraffin-embedded liver tissue were dewaxed by washing in xylene and rehydrated through a gradual series of ethanol and distilled water. Proteinase K permeabilized sections were subjected to enzymatic in situ labeling of DNA strand breaks using TUNEL-technique. The DNA strand breaks were revealed by adding alkaline phosphatase (AP)-converter with subsequent staining with NTB/BCIP solution as the chromogenic substrate. DNA-se pretreated tissue sections were used as a positive-control for apoptotic cells detection by TUNEL-assay. After counterstaining with eosin, sections were analyzed by light microscope (objective 20X). The dark-stained (TUNEL+) nuclei of liver cells were counted in randomly selected 10 histological fields per section. Enormously high numbers of TUNEL+ cells were present in DNA-se pretreated sections, whereas their numbers were considerably low (or even absent) in the control and in Cd-affected liver sections (Fig. 1). The nonparametric Kruskal-Wallis test was used for comparison between groups. Results were expressed as mean ± SEM. Statistical significance was set at p<0.05. Agarose gel (2% w/v) electrophoresis of DNA preparations extracted from paraffin embedded liver tissue was carried out according to the protocol described in (11). Gels were stained with ethidium bromide (5 microgram/ml) for 10 min and photographed under UV light. DNA extracted from dexamethasone (5 mg/kg from Krka, Slovenia) treated 18-day-old mice thymuses was used for electrophoretic detection of internucleosomal 200 bp fragments typical for apoptotic breakdown of DNA (12).

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**Fig. 1.** TUNEL reaction in mouse liver specimens  
A. DNA-se pretreated mouse liver as a positive control. Most cells exhibit dark-stained, TUNEL+ nuclei (original magnification ×40). B. Negative control obtained by omitting the terminal deoxynucleotidyl transferase enzyme from the TUNEL reaction; all cells are TUNEL- (original magnification ×20). C. TUNEL+ apoptotic hepatocytes with dark-stained nuclei (arrows) in the liver specimen of a mouse after 2 hours of intoxication by CdCl₂ (original magnification ×20).

**Results**

The activities of tRNA – one of the key elements of priming stage of translation machinery – isolated from normal (control) mice liver and mice liver 2 hours, 8 hours, and 24 hours after intoxication with sublethal dose of Cd (1.6 mg Cd²⁺ per 1 kg of body mass) were compared in vivo. The effect of Cd intoxication on the acceptor activity of tRNA⁶⁰ as a representative of all tRNA families is shown in Fig. 2. After 2 hours, 8 hours, and 24 hours of Cd intoxication, the acceptor activity of tRNA⁶⁰ was reduced by 43%, 73%, and 54%, respectively.

Next, we examined influence of Cd²⁺ ions on the activity one of the 20 aminoacyl-tRNA synthetases, namely leucyl-tRNA synthetase. The results obtained indicate that activity of leucyl-tRNA synthetase was suppressed down to 81%, 81%, and 70% of the control level 2 hours, 8 hours, and 24 hours after injection of CdCl₂ solution, respectively (Fig. 3).

In order to evaluate direct effects of Cd²⁺ ions on tRNA⁶⁰ and on leucyl-tRNA synthetase, the experiments in vitro were performed. CdCl₂ solution was added into the reaction mixture with preparations of tRNA and aminoacyl-tRNA synthetases isolated from control mice liver. In reaction mixture, final concentrations of Cd²⁺ ions were 5 µM, 10 µM, 15 µM, and 20 µM. Acceptor activity of tRNA⁶⁰ and activity of leucyl-tRNA synthetase were measured (Fig. 4). These experiments showed that 10 µM concentration of Cd²⁺ ions in reaction mixture decreased the acceptor activity of mice liver tRNA⁶⁰ and activity of leucyl-tRNA synthetase by 40%. Higher concentrations of Cd²⁺ ions (20 µM) almost fully inactivated the tRNA⁶⁰, whereas partially reduced activity of leucyl-tRNA is restored.

![Graph](image)

**Fig. 2.** Acceptor activity of tRNA⁶⁰, isolated from mouse liver in norm and 2 hours, 8 hours, and 24 hours after injection of CdCl₂ solution (1.6 mg Cd²⁺ per 1 kg of body mass)

The data represents results of 8–14 separate experiments. Differences between control and experimental groups are statistically significant.

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Fig. 3. Leucyl-tRNA synthetase activity of mice liver post-mitochondrial supernatant in norm and 2 hours, 8 hours, and 24 hours after injection of CdCl$_2$ solution (1.6 mg Cd$^{2+}$ per 1 kg of body mass)

Data represent results of 8–10 separate experiments. Differences between control and experimental groups are statistically significant.

synthetase was 30% of the control activity (Fig. 4).

How Cd-induced partial inactivation of the components of translation in vivo correlates with liver cell viability, we assessed according to the effect of Cd ions on apoptotic cell number in paraffin-embedded liver tissue sections. We have determined that 24 hours after CdCl$_2$ injection, the number of TUNEL positive cells did not differ from the control range (Fig. 5). Agarose-gel electrophoresis of genomic DNA isolated from Cd-affected mice liver revealed extensively degraded DNA (Fig. 6 A, lane 3). In specimens from the control liver, DNA was concentrated at the top of the lane or displayed large fragments containing more than 2652 bp (Fig. 6, lane 2). Although, there were small numbers of TUNEL positive cells detected in both the control liver and Cd-affected liver, no typical apoptotic ladder of DNA was reveal by electrophoresis (Fig. 6 A, lanes 2 and 3). Extensively degraded DNA

Fig. 4. Activities of mouse liver tRNA$^{Leu}$ and leucyl-tRNA synthetase after treatment with CdCl$_2$ in vitro

Activities were determined in the presence of indicated concentration of Cd$^{2+}$. The data represents results of 6–8 separate experiments.
**Fig. 5.** The number of TUNEL-positive cells in mouse liver 24 hours after injection of CdCl₂ solution (1.6 mg Cd²⁺ per 1 kg of body mass)

Cell counting was carried out in 10 histological fields in each of 3 sections taken from experimental and control liver. The data represents results of 3 separate experiments.

indicates on non-apoptotic cell death type (presumably necrosis) as a dominant form of the liver cell death after 24h of Cd exposure. It is noteworthy, that manifestation of non-apoptotic cell death coincided with a significant diminution of protein synthesis rate observed in 24 hours of the liver exposure to Cd. Validity of the data of DNA electrophoresis was verified using preparations of DNA extracted from thymuses of dexamethasone treated 18-day-old mice. Small DNA fragments (about 200 bp) revealed by electrophoresis can be considered as an evidence of active apoptosis of thymocytes (Fig. 6 B, lane 2). Thus, these data confirm reliability of the qualitative detection of state of DNA extracted from Cd-intoxicated mice liver by agarose-gel electrophoresis.

**Discussion**

Being a ubiquitous toxic metal Cd causes deterioration of a number of cellular processes. Our previous investigations have revealed initial inhibition of translation in mice liver within the first 2–4 hours, which progressed into stimulation, reaching its maximum at 8th hour, and subsequent decrease at 24th hour after injection of a single dose of CdCl₂ (1.6 mg per 1 kg of body mass) (6). Notable, that within 24 hour period after a single injection of CdCl₂ solution (1.6 mg per kg of body mass), Cd²⁺ ions are being accumulated in liver in a concentration as high as 14.96 μg/g, what corresponds to 0.134 μmol/g (13). *In vitro* Cd²⁺ at 40 μM concentration exerted stimulatory effect on both parameters of translation – the rate and the level, while at 60 μM concentration the ion caused sharp diminution of those parameters (6). In present study, we focused on evaluation of Cd²⁺ ion effects on the translation process and on the activity of key components of liver translation machinery as well as on liver cell viability. Bearing in mind the fact that the translation rate in mouse liver was maximum at 8h after Cd intoxication (6), the decreases in activities of both studied translation machinery components (tRNA⁰ and leucyl-tRNA synthetase) (Fig. 2 and 3) seem to be paradoxical at this time point of Cd exposure. These seemingly controversial results can be interpreted in terms of Cd-induced synthesis of cytoprotective proteins such as metallothionein (3) and heat-shock proteins known as chaperons (4). Active synthesis of those proteins can contribute to an increase of the translation rate in mouse liver after 8h of Cd intoxi-

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**Fig. 6.** Agarose-gel electrophoresis of DNA extracted from the mice liver (A) and from dexamethasone-treated mice thymuses (B)

In A, the lane 1 – DNA molecular weight marker XIII (Roche), the lane 2 – DNA extracted from control mice liver, the lane 3 – DNA extracted from 24 hour Cd-intoxicated mice liver. In B, the lane 1 – DNA extracted from dexamethasone-treated mice thymuses, the lane 2 – the molecular weight marker GeneRuler 100 bp DNA Ladder plus.
cation reported in our recent study (6). It is known that Cd ions directly react with nucleic acids (14) and with sulfhydryl groups of proteins (1). In the present in vivo study, the direct effects of Cd ions might cause inhibition of the activities of tRNALeu and leucyl-tRNA synthetase. Absence of the positive correlation between changes in the translation rate and the activities of tRNALeu as well as leucyl-tRNA synthetase observed in our experiments may be explained by peculiarities of the regulation of translation: neither tRNA nor aminoacyl-tRNA synthetases do not exert control over the rate of protein synthesis under Cd intoxication. The process is probably controlled by other components of translation machinery.

Cd complexation with protein sulfhydryl groups may disrupt cellular redox state (8) and inhibit a number of enzymes, thereby impairing viability of cells. As Cd ions accumulate in the liver and the kidney, cells of these organs are the primary targets for toxic action of Cd (8). Irreversible damage of vitally essential cellular processes can lead to cell death. According to our recent investigations, Cd intoxication up to 8 hours results in an increase of apoptotic (TUNEL+) cell number in mouse liver sections (15). Cd seems to induce apoptosis via a mitochondria-dependent pathway (16). Pro-apoptotic Cd effect can be potentiated by a mild decrease of reduced glutathione level in the transformed rat liver cells (8). As Cd induces necrosis under conditions of sulfhydryl group deficiency, oxidation of glutathione appears to be critical for changing of the cell death phenotype from apoptosis to necrosis. Cd-caused decrease of reduced glutathione level is intoxication time and dose dependent (17). These findings coincide with our results that have revealed the same number of TUNEL+ cells in both the control liver and the liver of mice subjected to 24h of Cd intoxication (Fig. 5). Although, TUNEL+ cells were identified in both the control and the Cd-affected liver, DNA electrophoresis did not reveal DNA-laddering characteristic to apoptosis. This can be caused by relatively low number of apoptotic cells that is insufficient for detection of apoptosis-specific DNA fragments by electrophoresis (18). There was the “smear” background pattern found by DNA electrophoresis (Fig. 6A, lane 3), which has been suggested to indicate extensive degradation of nuclear DNA. This can be assumed as an evidence of necrosis induced by 24h exposure of the liver to Cd ions. Other authors also reported about Cd-caused non-apoptotic cell death (9, 18). Manifestation of necrosis might be a consequence of coincidence of several different events including Cd-caused inhibition of mitochondria (20), down-regulation of antioxidant enzymes expression (21), and inhibition of activity of antioxidant enzymes (22). Thus, depletion of ATP, inhibition of protein synthesis, and uncontrolled oxidative stress may be regarded as the main causes of Cd hepatotoxicity.

Conclusions

1. Cadmium ions significantly reduce activities of both, tRNA and aminoacyl-tRNA synthetase, what do not positively correlated does with the changes of total protein synthesis in mice liver.

2. There is no significant difference between the number of apoptotic cells in the control liver specimens and in those after 24 hours of intoxication with cadmium chloride. In latter specimens DNA electrophoresis revealed as extensive degradation of DNA as in the cell necrosis.

Kadmio jonų poveikis pelės kepenų baltymų sintezės pradinei stadijai ir įstastelių žūčiai

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Raktažodžiai: kadmis, tRNR, leucil-tRNR-sintetazė, apoptozė, nekrozė.

Santrauka. Darbo tikslas. Įvertinti kadmio jonų poveikį pelių kepenų tRNRLeu ir leucil-tRNR-sintetazės aktyvumams in vivo ir in vitro bei nustatyti kadmio jonų indukuotą kepenų įstastelių žūties tipą.

Tyrimo medžiaga ir metodai. Intoksikacija kadmio jonas sukelta baltons laboratorinėms pelėms į pilvo ertną sušvirkštus kadmio chlorido tirpaluo (1,6 mg kadmio jonų 1 kg kūno masės). Suminiai tRNR preparatai išskirti pridedant etanolio ir izopropanolio į supernatantą, gautą fenolio deproteinizavus kepenų homogenatą. Leucil-tRNR-sintetazės aktyvumas nustatytas kepenų bemitochondriniame supernantante. tRNRLeu ir leucil-tRNR-sintetazės aktyvumai įvertinti pagal [14C]-leucino panaudojimą tRNR aminoacilinimo reakcijos metu. Įstastelių žūties fenotipas tirtas imunohistochemiškai panaudojant TUNEL in situ fermenting reakciją.

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