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Original article

Enzymatic antioxidant defense in isolated rat hepatocytes exposed to cadmium

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Abstract

The aim of the study was the evaluation of cadmium effects on the activity of antioxidant enzymes in rat hepatocytes. The studies were conducted with isolated rat hepatocytes incubated for 1 or 2 hours in a modified (deprived of carbonates with phosphates) Williams' E medium (MWE) in the presence of cadmium chloride (25, 50 and 200 μM). Hepatocytes incubated in the MWE medium without cadmium chloride were used as a control. The application of the modified Williams' E medium allowed for the appearance of cadmium compounds in a soluble form that is indispensable for suitable estimation of its toxic action. There were evaluated markers of the oxidative stress such as: concentration of thiobarbiturate reactive substances (TBARS) – proportional to the level of lipid peroxidation, concentration of reduced glutathione (GSH), and the activity of antioxidant enzymes, including superoxide dismutase (SOD1 and SOD2), catalase (CAT), total glutathione peroxidase (GSHPx), selenium – dependent glutathione peroxidase (SeGSHPx), glutathione transferase (GST) and glutathione reductase (GSHR). Alterations of antioxidant enzymes activity, the level of TBARS and GSH in isolated rat hepatocytes caused by cadmium *in vitro,* were shown to depend on the concentration and time of exposure of cells to this metal. The increased level of TBARS and GSH was observed as well as changes in the activity of antioxidant enzymes. The activity of SOD isoenzymes and CAT was increased, whereas GSHPx and GST were decreased. These results indicate that cadmium induces oxidative stress followed by alterations in the cellular antioxidant enzyme system in isolated rat hepatocytes.

Key words: cadmium; isolated rat hepatocytes; oxidative stress; lipid peroxidation (TBARS); reduced glutathione (GSH); antioxidant enzymes

Introduction

Cadmium (Cd) is an environmental contaminant, comprising a risk factor for human and animal health. It is present in air, soil, water and in plant and animal tissues (Järup et al. 1998). In humans cadmium accumulates mainly in kidney and liver (Kołacz et al. 1996). Lungs, bones, and the immunological system are also targets for this metal toxicity (Brzóska et al. 1998). It is well known that cadmium acts in a nephrotoxic, hepatotoxic and neurotoxic manner, and also has distinct effects of mutagenic and carcinogenic

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character. Cadmium is classified as an environmental factor with carcinogenic effect in humans (category A1) (IARC 1993). Exposition to cadmium leads to the induction of kidney, pancreatic, lung and prostate tumors (Waalkes 2003, Waisberg et al. 2003, EFSA 2009).

Despite intensive studies, the mechanism of cadmium toxicity in organisms has not been clarified yet. Cadmium toxicity might be a result of interactions with zinc (Zn), copper (Cu), iron (Fe) and selenium (Se). Those metals are replaced by cadmium in the metalothionein complexes. Cadmium also binds to reactive residues in biologically active molecules (i.e. -SH, -OH, -COOH, $-NH_2$, $-PO_3H_2$), where N and O atoms are electron donors (Pourhammad and O'Brien 2000). Up to date different mechanisms were indicated: cadmium might impair the redox state and in consequence cause the induction of oxidative stress, weakening of antioxidant system, resulting in loss of cell membranes integrity (Shaikh et al. 1999, Stohs et al. 2001, Pourhammad et al. 2003).

The aim of this work was to determine the influence of cadmium on the activity of antioxidant enzymes in isolated rat hepatocytes. Since liver is exposed to both the accumulation and toxic action of cadmium, isolated rat hepatocytes were used as an alternative research model *in vitro*, commonly accepted as suitable for studies on xenobiotics metabolism, hepatotoxicity, and general cytotoxicity. Freshly isolated hepatocyte suspensions reflect the metabolic capabilities of hepatocytes *in vivo.*

Materials and Methods

Chemicals

Antibiotics (penicillin G, streptomycin, amphotericin B), 0.4% solution of trypan blue (TB), dimethylosulphoxide (DMSO), type IV colagenase, Williams' E Medium (WE), fetal bovine serum (FBS), and cadmium chloride ($CdCl_2$) were obtained from Sigma Chemicals Co (ST. Luis, MO, USA). Reagents used in the study were obtained from: RANSOD kit from Randox, United Kingdom. Cadmium chloride, 5,5'-dithio-bis-2-nitrobenzenic acid (DTNB), reduced glutathione (GSH), oxidized glutathione (GSSG), thiobarbituric acid (TBA), sulphosalicylic acid, hydrogen peroxide, cumen peroxide, glutathione reductase, and Tris base were purchased from Sigma. Catalase and xanthine were obtained from ICN Biomedicals Ltd, ammonium molibdate from Wampur Company, Tween 20 from Promega, NADPH from MP Biomedicals LCC. All inorganic salts and other reagents used in research were purchased from POCh company (Polish Chemical Reagents).

Animals

Male Wistar rats (n-3) were used in our studies (body weight 200-250 g). Rats were housed in plastic cages under standard laboratory conditions (12 h light/dark cycle, 22±1°C, humidity 65±10%) and allowed free access to standard laboratory rat chow and water *ad libitum.* Animals were allowed two weeks quarantine before the experiments. During this period animals were provided with water and solid feed (Ssaniff® R/M-H, Saniff Spezialdiaten GMBH, D-59494 Soest, Germany).

Isolation and incubation of hepatocytes with cadmium chloride ($CdCl_2$)

After 2 weeks (required to adapt to laboratory conditions), hepatocytes were isolated from rat livers according to INVITOX No 20 protocol (Invitox protocol nr 20 1991), which includes: surgery (cannulation of the portal vein, and inferior vena cava), two-step liver perfusion (*in situ* and *in vitro* after liver isolation), achieving a suspension of isolated hepatocytes, and its qualitative and quantitave evaluation.

Surgery procedure was performed under deep general anesthesia achieved by intramuscular injection of ketamine (Narkamon 5%, Spofa Praha), 100 mg per kg of body weight, and xylazine (Rometar 2%, Spofa Praha), 20 mg per kg of body weight. Directly after liver isolation, animals were killed by intersection of the cervical spinal cord.

Cells were used for the experiments if their viability determined by trypan blue test (Tennant 1964) exceeded 85%.

Isolated hepatocytes ($1x10^6$ of living cells/ml) were incubated for 2 hours in the modified (deprived of phosphates and carbonates) Williams' E medium (MWE), pH 7.35, with $CdCl_2$ at the concentrations: 25, 50, 200 μM, respectively. Controls were the hepatocytes incubated in the MWE medium without $CdCl_2$ (0 mM). Incubation for 1-2 hours corresponds to a single exposure to cadmium *in vivo*.

In the present work a medium for preparing hepatocytes suspension composed as described by Borzecka et al. (2004) and Bojakowska (2006) was applied. This guarantees that cadmium compounds remain in a soluble form, which is available for cells and therefore able to exert toxicity. For this purpose we used the modified MWE medium, deprived of carbonates and phosphates, which form hardly soluble salts with cadmium.

Hepatocytes were incubated in the Erlenmayer flasks, in a water bath, at 38°C, under continuous agitation. During incubation, the hepatocytes suspension was saturated with carbogen (5% CO_2 + 95% O_2). Samples for analysis were collected after 1 or 2 h of

incubation. All experiments were conducted in triplicate.

The initial procedures involving living animals (rats) followed the provision of the Animal Protection Act and were approved by Local Ethical Committee for Animal Experiments, Division of Pharmacology and Toxicology, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences – SGGW (decision No. 16/2006).

Biochemical analysis

After 1 and 2 h of incubation the markers of oxidative stress were determined in the hepatocytes suspension, such as the concentration of thiobarbiturate reactive substances (TBARS) – proportional to level of lipid peroxidation, and concentration of reduced glutathione (GSH), and the activity of antioxidant enzymes: superoxide dismutase (SOD1 and SOD2; EC 1.15. 1.1), catalase (CAT; (EC 1.11.1.6), total glutathione peroxidase (tot. GSHPx; EC 1.11.1.9), selenium-dependent glutathione peroxidase (SeGSHPx; EC 1.11.1.9), glutathione transferase (GST; EC 2.5.1.18) and glutathione reductase (GSHR; EC 1.6.4.2.). The protein concentration was also determined in hepatocytes according to the method described by Bradford (1976).

Oxidative stress markers

To evaluate if $CdCl_2$ induces oxidative stress in hepatocytes, the lipid peroxidation level and concentration of reduced glutathione (GSH) were measured.

Lipid peroxidation levels were determined according to the method described by Ohkawa (Ohkawa et al. 1979). This method employs the measurement of the concentration of thiobarbiturate reactive substances (TBARS) such as malondialdehyde (MDA) and other secondary lipid peroxidation products.

The concentration of reduced glutathione (GSH) was estimated according to the method described by Ellman as well as by Sedlak and Lindsay (Ellman 1959, Sedlak and Lindsay 1968). In this method a colored product is generated in reaction of GSH with 5,5̀-dithio-bis-nitrobenzoic acid (DTNB).

Antioxidant enzymes activity

The activity of copper-zinc superoxide dismutase (SOD1) was determined by means of RANSOD kit produced by Randox, UK. The activity of manganese superoxide dismutase (SOD2) was determined according to the method described by Beauchamp and Fridovich, modified by Oberley and Spitz (Beauchamp and Fridovich 1971, Oberley and Spitz 1984). Catalase activity (CAT) was measured according to the method described by Goth (1999). The total activity of glutathione peroxidase (tot. GSHPx) was assayed according to the method described by Wendel (1981). The activity of selenium-dependent glutathione peroxidase (SeGSHPx) was determined according to the method described by Wendel, and Paglia and Valentine (Paglia and Valentine 1967, Wendel 1981). Glutathione transferase (GST) activity was determined according to the method described by Habig et al. (1974). The activity of glutathione reductase (GSHR) was determined according to the method described by Goldberg et al. (1983). The activity of all studied enzymes was expressed in U/mg of protein. Concentrations of GSH and TBARS were expressed in μmol/mg of protein, and nmol/mg of protein, respectively.

All biochemical parameters were measured at the Department of Biochemistry, Warsaw Medical University.

Statistical analysis

All results are expressed as means of 3 independent experiments ± SD. The significance of differences was calculated by t-Student test. Differences were considered significant if $p \leq 0.05$. Statistical evaluation of the results was performed using the programme Statistica 6.0.

Results

TBARS and GSH concentration in the isolated rat hepatocytes exposed to cadmium chloride are shown in Fig. 1 and 2.

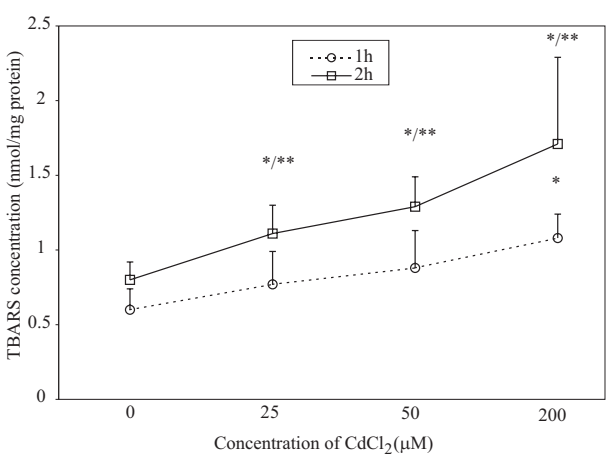

Fig. 1. Effect of $CdCl_2$ on lipid peroxidation levels in isolated rat hepatocytes.
The control were hepatocytes incubated in MWE medium without $CdCl_2$ (0μM). Data were expressed as means ± SD (n-3)
* – significant versus control ($p \leq 0.05$)
** – significant versus 1 hour exposure of hepatocytes to $CdCl_2$ ($p \leq 0.05$)

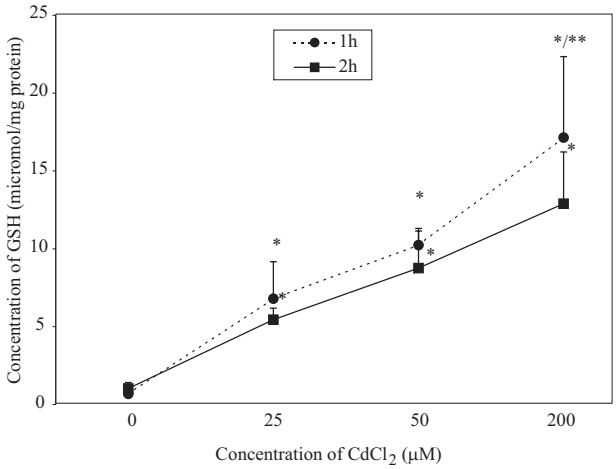

Fig. 2. Effect of $CdCl_2$ on GSH levels in isolated rat hepatocytes.
The control were hepatocytes incubated in MWE medium without $CdCl_2$ (0μM). Data were expressed as means ± SD (n-3)
* – significant versus control (p≤0.05);
** – significant versus 1 hour exposure of hepatocytes to $CdCl_2$ (p≤ 0.05)

The exposure of the isolated hepatocytes to cadmium chloride (25, 50, 200 μM) elevated TBARS levels. TBARS increased in a dose- and time-dependent manner. In hepatocytes incubated for 1 h, a significant increase of TBARS levels was observed only in cells exposed to 200 μM $CdCl_2$. Longer exposure (2 h) of hepatocytes to cadmium chloride resulted in a distinct increase of TBARS level. A significant increase of TBARS levels was observed already in hepatocytes exposed to the lowest dose of cadmium chloride used in this experiment (25 μM). Under these conditions the levels of TBARS were also significantly higher than in hepatocytes exposed to 25 μM $CdCl_2$ for 1 h. Incubation of hepatocytes with $CdCl_2$ at higher concentrations (50 and 200 μM) resulted in an additional increase of TBARS level. The highest level of TBARS was observed in hepatocytes incubated for 2 h in the presence of 200 μM $CdCl_2$ (Fig. 1).

The exposure of hepatocytes to $CdCl_2$ resulted in a distinct increase in the GSH. Significant increase of GSH levels was already observed for 25 μM $CdCl_2$. Higher concentrations (50 and 200 μM) of $CdCl_2$ increased the GSH levels even more. However, the GSH levels in hepatocytes incubated with $CdCl_2$ for 2 h were lower than in hepatocytes incubated for 1 h, but significant differences were observed only for hepatocytes exposed to 200 μM $CdCl_2$ (Fig. 2).

Activities of antioxidant enzymes are presented in figures 3 and 4. Significant increases of SOD1 and SOD2 activity in hepatocytes were observed in cells exposed to 50 and 200 μM $CdCl_2$ for 1 or 2 h. The highest activity of both SOD isoenzymes was found in hepatocytes exposed to 200 μM $CdCl_2$. Significant time-dependent differences in SOD activity were observed only for SOD1 in hepatocytes exposed to 200 μM $CdCl_2$ (Fig. 3a).

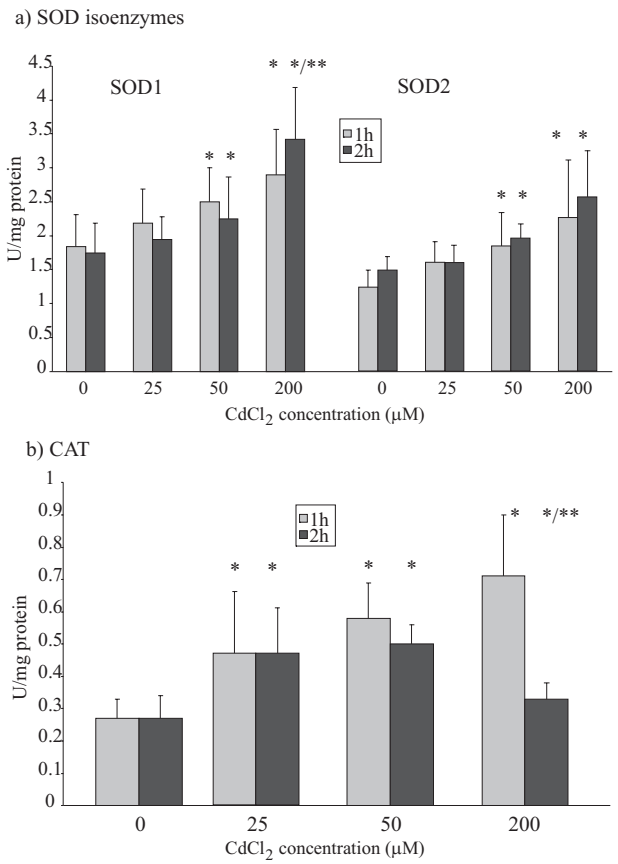

Fig. 3. Effect of $CdCl_2$ on SOD isoenzymes and CAT activity.
The control were hepatocytes incubated in MWE medium without $CdCl_2$ (0μM).
Data were expressed as means ± SD (n-3)
* – significant versus control (p≤ 0.05)
** – significant versus 1 hour exposure of hepatocytes to $CdCl_2$ (p≤ 0.05)

In hepatocytes exposed to $CdCl_2$, the activity of CAT was significantly increased already at the concentration of 25 μM, independent from exposure time. One hour incubation of hepatocytes with 50 and 200 μM $CdCl_2$ resulted in a significant dose-dependent increase of CAT activity, whereas CAT activity in hepatocytes exposed for 2 h to 200 μM $CdCl_2$ was notably lower than in hepatocytes exposed for 1 h (Fig. 3b).

The activity of total GSHPx and SeGSHPx in hepatocytes exposed for 1 or 2 h to $CdCl_2$ (25, 50, 200 μM) did not differ significantly from the control. Changes of these enzyme activities were more distinct in a time-dependent manner. In hepatocytes exposed for 2 h to $CdCl_2$, the activity of total GSHPx was significantly lower than in hepatocytes exposed for 1 h to all concentrations. Whereas the activity of SeGSHPx was decreased only in cells exposed to 200 μM $CdCl_2$

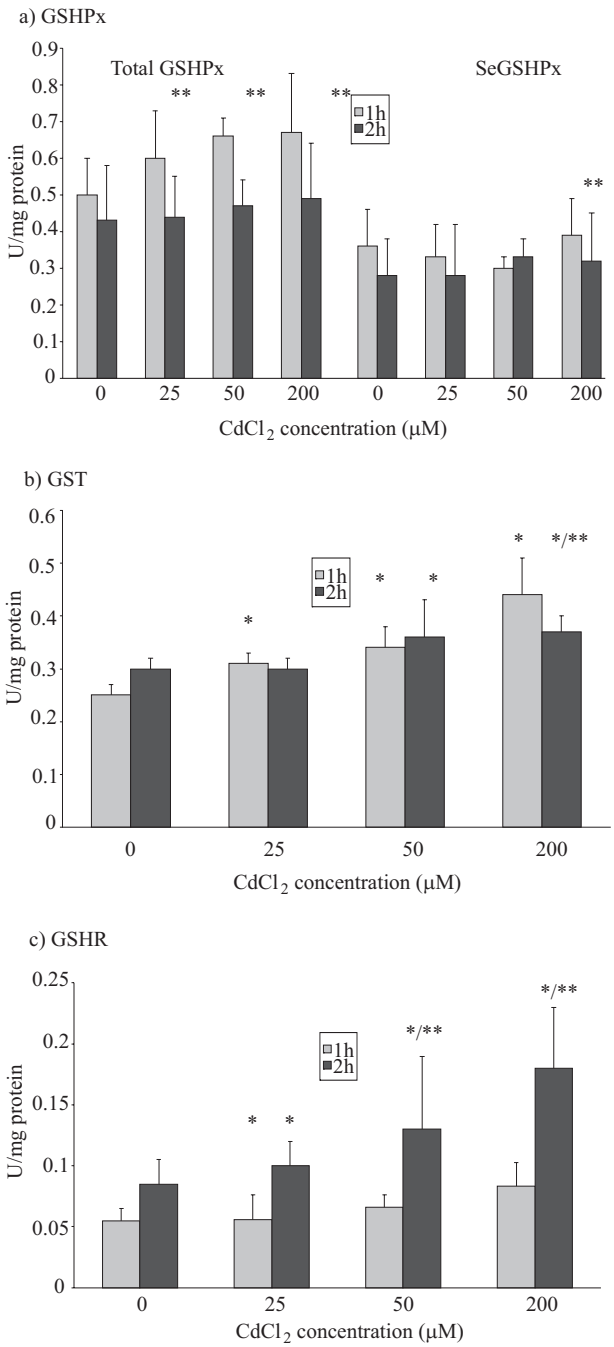

Fig. 4. Effect of $CdCl_2$ on activity of GSH-dependent enzymes.
The control were hepatocytes incubated in MWE medium without $CdCl_2$ (0μM).
Data were expressed as means ± SD (n-3)
* – significant versus control ($p \leq 0.05$)
** – significant versus 1 hour exposure of hepatocytes to $CdCl_2$ ($p \leq 0.05$).

for 2 h, but its activity was still higher than in controls (Fig. 4a). GST activity in hepatocytes exposed for 1 or 2h to $CdCl_2$ was higher than in the control. After 1 h of incubation a significant increase of GST was observed already at 25 μM $CdCl_2$, and after 2 h exposure at 50 μM concentration of $CdCl_2$. The highest activity of GST was found after 1 h exposure of hepatocytes to 200 μM $CdCl_2$. However, 2 h incubation of hepatocytes with 200 μM $CdCl_2$ resulted in a significant decrease of the GST activity (Fig. 4b).

In hepatocytes incubated for 1 h with $CdCl_2$, significant changes of the GSHR activity was observed only in cells exposed to the highest concentration of 200 μM. 2 h exposition to $CdCl_2$ resulted in a significant dose-dependent increase of the GSHR activity. A significant increase was observed already at 25 μM (Fig. 4c).

Discussion

The mechanisms involved in cadmium toxicity are not well understood yet. Previous studies showed that cadmium toxicity is a result of different mechanisms, one of them being the induction of the oxidative stress. However, cadmium does not directly induce the formation of reactive oxygen species (ROS), but affects antioxidant enzymes and low molecular weight antioxidants that have a significant role in cellular homeostatis (Fariss 1991, Shaikh et al. 1999, Jurczuk et al. 2004).

The results presented here confirm the induction of the oxidative stress and weakening of cellular antioxidant defense mechanisms. This is evidenced by increased lipid peroxidation levels and a distinct increase of the GSH level in hepatocytes exposed for 1 or 2 h to different concentrations of $CdCl_2$. For both oxidative stress markers time- and dose- dependent effects were observed.

Increased levels of lipid peroxidation in the isolated hepatocytes exposed to $CdCl_2$ indicated elevation of generation of ROS . It is probably due to releasing of transition metals by cadmium from their place of occurrence in cells. Binding of cadmium with ceruloplasmine or ferritine in the liver, might displace ferric or copper ions, which than participate in Fenton reactions forming the most reactive hydroxyl radicals (Brzóska et al. 1998, Pourhammad and O'Brien 2000, Stohs et al. 2001, Waisberg et al. 2003). Cadmium might also damage mitochondria, leading to the release of Fe^{2+} from enzymatic complexes of respiratory chain. Another possibility of ROS formation is the inhibition of the respiratory chain III complex, responsible for electron transport from ubiquinone to cytochrome c (Casalino et al. 2002, Wang et al. 2004). The increase in the GSH levels in hepatocytes exposed for 1 or 2 h to cadmium points to an active participation of GSH in the cellular defense against ROS. Whereas the decrease of the GSH levels in hepatocytes exposed for 2 h to the highest concentration of $CdCl_2$ indicated oxidative stress in these cells. In the study it was observed that $CdCl_2$ at high concentrations and after a longer exposure (2 h) depletes the GSH levels in the isolated rat hepatocytes, which

contributes to the increased ROS formation. These results agree with the observations by Pourhammad and O'Brien (Pourhammad and O'Brien 2000).

It is known that the formation of large amounts of ROS in hepatocytes leads to the depletion of antioxidant mechanisms and to a decrease of defensive ability of cells (Jurczuk et al. 2004). Hepatocytes have a limited ability to survive the oxidative stress (Pourhammad et al. 2003). Insufficiency of the antioxidant system decreases the capability to inactivate ROS in liver cells, leading to the loss of integrity. Our results clearly show that the exposure of isolated hepatocytes to $CdCl_2$ leads to such a decrease of their antioxidant potential.

The increase of SOD1 and SOD2 activities in hepatocytes observed after 1 and 2 h exposure to $CdCl_2$ (especially at higher concentrations) indicated the activation of antioxidant defense mechanisms and therefore adaptation of liver cells to survive in the oxidative stress conditions. A higher activity of catalase found in hepatocytes exposed for 1 h to cadmium, suggests defense against an excess amount of hydrogen peroxide produced by SOD. The decrease of CAT activity after 2 h of exposure to 200 μM cadmium suggests inhibition of catalase activity, probably due to the release of Fe^{2+} from the prosthetic group of the enzyme (Casalino et al. 2002). Two hours incubation of hepatocytes with $CdCl_2$ at high concentration caused not only a decrease of the GSH levels (in comparison with 1 h incubation), but also a decrease of the activity of the GSH dependent enzymes such as total GSHPx, SeGSHPx, and GST, which are responsible for scavenging of hydrogen peroxide, organic peroxides, or endo- and egzogenic electrophilic compounds (Hayes and McLellan 1999). Oxidative stress in hepatocytes exposed to cadmium was also indicated by an increased activity of GSHR, the enzyme responsible for the regeneration of the GSH form.

In physiological conditions the GSH acts as cell "thiol buffer", which very efficiently counteracts the effects of -SH residues oxidation. It prevents an impairment of the biological activity of many functional cell proteins. Confirmation of this effect were the decreased GSH levels and similar changes of CAT and Se-GSHPx activity in hepatocytes exposed for 2 h to 200 μM $CdCl_2$. Binding of cadmium with GSH decreases its amount in hepatocytes, impairing the reactions of GSH redox cycle enzymes, where GSH is a co-substrate.

The depletion of GSH in cells leads to peroxidation of lipids and proteins (Hayes and McLellan 1999, Sies 1999, Sen 2000, Pastore et al. 2003).

Nevertheless, if cadmium directly, or indirectly, induces the oxidative stress in hepatocytes, this leads to changes of the structure of macromolecules such as DNA, proteins, lipids and other and, in consequence, to their irreversible damage and disturbance of biological function. Lipid peroxidation causes not only the loss of cell membranes integrity, but also impairs the ion gradient across membrane, and modifies the activity of many membrane proteins (receptors, ionic channels) (Kehrer 1993, Williams and Jeffrey 2000). Oxidative stress induced in hepatocytes by cadmium might be exacerbated due to weakening of cell reducing potential caused by the inhibition of antioxidant enzymes. Inhibition of the activity of antioxidant enzymes by cadmium might indirectly participate in oxidative damage of proteins, lipids or DNA. Toxicity of cadmium might also be related to the impairment of signaling pathways, and induction of apoptosis (Sen 2000, Robertson and Orrenius 2000). The ability of cadmium to induce the oxidative stress, which causes the DNA oxidative damage, explains the potential role of this metal in the onset of lung, prostate, pancreatic and other tumors (Waalkes 2003, Waisberg et al. 2003).

The application of the modified William's medium (deprived of carbonates and phosphates) allowed for evaluation of cadmium toxicity. This was confirmed by the research under the same experimental conditions (medium MWE, 2 h exposition to $CdCl_2$) in rat hepatocytes cultivated in monolayer in 96-well cell culture plates. Cytotoxic effects of cadmium were noted after cell exposure to cadmium chloride at several times lower concentration; a statistical significant decrease of MTT (78±16%) and AlamarBlue (44±8%) reduction rate were already observed after cell exposure to cadmium chloride at the concentration of 6.25 μM. The exposure of cells to higher cadmium concentrations resulted in the further decrease of the MTT reduction. The exposure of rat hepatocytes to 25, 50, 100 and 200 μM of $CdCl_2$ resulted in the decrease of the MTT reduction to 53±26%, 36±13%; 24±14% and 9±1%, respectively. It should be emphasised that hepatocytes incubated in a standard WE medium showed a markedly higher MTT reduction rate (87±6%, 75±15%, 53±24% and 25±12%), respectively in the presence of cadmium at the concentration of 25, 50, 100 and 200 μM. Noteworthy, the MTT reduction rate was still high (53±24%) in the presence of 100 μM of $CdCl_2$. The cytotoxicity of $CdCl_2$ based on calculated EC50 values in hepatocytes incubated in WE was 2.5 times lower than in hepatocytes incubated in MWE. The EC50 values for $CdCl_2$ calculated on the basis of MTT and Alamar Blue reduction rate was 132.5±66.5 and 66.2±0.2 μM for cells incubated in WE and 46.8±13.9 and 26.1±3 μM for cells incubated in MWE, respectively (Borzęcka et al. 2004, Bojakowska 2006).

Conclusions

The increased lipid peroxidation, changes of GSH concentration and antioxidant enzymes activity in the isolated rat hepatocytes caused by cadmium suggest

the impairment of antioxidant defense mechanisms as one of the most prominant effect the $CdCl_2$ toxicity in hepatocytes.

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