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The Response of Huh 7 Cells Scavenger Enzymes to Cadmium

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Abstract

Prolonged exposure to cadmium is known to have diverse toxic effects on cells of different organs. We have investigated the effects following administration of $CdCl_2$ 5 μM and 20 μM in Huh 7 cells. The results of this study revealed an increase in activities of the critical scavenging free radicals enzymes, catalase and glutathione peroxidase, indicating an oxidative stress response to cadmium administration. The impair in glucose-6-phosphate dehydrogenase activity, and its consequence, the diminution of the cellular NADPH content, had as a result the decrease in glutathione reductase activity. The alteration in the activity of enzymes involved in oxidative stress observed in our studies is likely to affect the capacity of Huh 7 cells to defend themselves and respond to cadmium-induced oxidative stress.

Keywords: cadmium, oxidative stress, catalase, glutathione peroxidase, glucose-6- phosphate dehydrogenase, glutathione reductase

Introduction

Cadmium (Cd) is an abundant and widely spread toxicant that is continuously accumulated in the environmental due to industrial activities. The ionic form of Cd (Cd^{2+}) is usually combined with ionic forms of oxygen (cadmium oxide, CdO), chloride (cadmium chloride, $CdCl_2$) or sulfur (cadmium sulfate, $CdSO_4$). Humans are exposed to Cd^{2+} primary through the ingestion of contaminated food [1] and the inhalation of cigarette smoke [2]. The exposure to Cd^{2+} on a chronic basis can cause adverse effects in the liver, kidney, brain, lung, pancreas, testis, placenta and bone [3,4,5]. Cadmium bioavailability, persistence and bioaccumulation make it potentially lethal [6].

The mechanism(s) for Cd toxicity are not well understood. It has been demonstrated that Cd exposure produces reactive oxygen species (ROS) [7], lipid peroxidation [8], DNA damage, and apoptosis [9] in various cell types.

ROS, such as hydroperoxyl, superoxide, and hydroxyl radicals, occur naturally under normal respiration or can be produced by exposure of tissue or cells to different conditions such as inflammation, hyperoxia, UV, ionizing irradiation, heavy metals, and certain oxidant chemicals [10]. Abnormal production of ROS can lead to the oxidative stress. Oxidative stress is a term used to describe an imbalance favoring prooxidants and/or disfavoring antioxidants, potentially leading to damage [11]. Cells have their own antioxidant defense mechanisms to neutralize the increased levels of ROS through antioxidant compounds such as vitamins C, E, reduced glutathione (GSH), ubiquinone [12], and various antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase [13,14]. The long term consequences of oxidative stress have been associated with the pathogenesis of a variety of

toxicities and diseases, including arteriosclerosis, diabetes, chronic inflammatory diseases, neurological disorders, and cardiovascular disease [15].

The aim of this study was to determine the magnitude of the oxidative stress generated by the treatment of Huh 7 cells with two different concentrations of $CdCl_2$. The antioxidant enzymes activities, including catalase (CAT), gluthatione peroxidase (GPX) and glutathione reductase (GR) were investigated. In addition, the activity of glucose-6-phosphate dehydrogenase (G6PD), the enzyme that regenerates the NADPH consumed in the GR-catalyzed reaction, was examined.

Materials and methods

Cell culture

The human hepatocyte carcinoma cells (Huh 7) were grown in Dulbecco`s Modified Eagle`s Medium (DMEM) containing 10% fetal bovine serum. Cells were plated at a density of 2×10^6 viable cells/culture flask ($75cm^2$). Cultures were maintained in a humidified 5% CO_2 air atmosphere at 37°C. Huh 7 cells were treated with 5μM and 20μM cadmium chloride ($CdCl_2$) and they were incubated at 37^0C for 4 hours.

Cd treatments and MTT Test

Cells were first harvested from culture flasks, washed and added to a flat-bottom 96-well plate at a density of 1×10^3cells in 200μl medium/well. The cells were further cultured for 24 hours and then treated with cadmium chloride ($CdCl_2$) to final concentrations of 2.5μM, 5μM, 10μM and 20μM. Triplicates for each concentration were exposed to $CdCl_2$ for 3, 4, 5, 6 and 8 hours, respectively.

The MTT test was used to test the viability of the cells. The medium was removed by aspiration, the cells were washed with 200μl of phosphate buffer solution (PBS)/well and then 50μl (1mg ml^{-1}) of MTT solution [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] was added on each well. After 2 hours of incubation, MTT was removed, and 50μl izopropanol were added. The absorbance at 595nm for each well was determined using a Tecan multireader (GENios)

Enzyme Assay

Huh 7 cells were harvested from culture flasks, washed with phosphate buffer solution (PBS) and centrifuged at 1,500xg for 10 min at 4^0C. Cell pellets were re-suspended in 1.5 ml of lysis buffer (20mM Tris-HCl, pH 7.5, 0.5mM PMSF, 0.3M NaCl and 0.2% Triton X-100) and then, ultrasonicated three times, for 30 seconds. The total extract was centrifuged at 3,000xg for 15min at 4°C. Aliquots of the supernatant were used for enzyme assays.

The CAT (EC 1.11.1.6) activity was determined by the Aebi method [16]. The disappearance of H_2O_2 was followed at 240nm. The catalase activity was calculated in terms of k/min/mg protein, where k is first order rate constant. The GPX (EC 1.11.1.9) activity was assayed according to the Beutler method [17]. The oxidation of NADPH was recorded at 340nm and 25ºC. The enzyme activity was calculated as U/mg protein, one unit been the amount of enzyme which oxidizes 1 nmole of NADPH per minute. The GR (EC 1.6.4.2) activity was recorded by Goldberg and Spooner method [18] and expressed as U/mg protein. One unit of GR activity was calculated as 1 μmol NADPH consummated per minute. The G6PD (EC 1.1.1.49) activity was assayed by the method of Löhr and Waller [19]. The rate of NADPH formation is a measure of the enzyme activity and it can be followed by means of the increase in extinction at 340nm. One unit of G6PD activity was expressed as 1 nmole of $NADP^+$ converted in NADPH per minute.

The protein estimation was done according to the method of Lowry et al. [20] using bovine serum albumin (BSA) as a standard.

All the spectrophotometric analyses were done with a Perkin-Elmer Lamda 25 Double Beam Spectrophotometer at 25ºC.

Statistical Analysis

All values were expressed as means ± SD. The differences between control and manganese-treated groups were compared by Student's t-test using standard statistical packages. The results were considered significant if the P value was less than 0.05.

Results and discussion

The activity of living cells, via mitochondrial dehydrogenases, was evaluated by MTT test. There were no changes observed in the cell viability after 3 and 4 hours of Cd^{2+} administration in different concentration to the Huh 7 cells (**Figure 1**). However, in prolonged treatment, for more than 4 hours, $CdCl_2$ induced cytotoxicity in this cells (**Figure 1**).

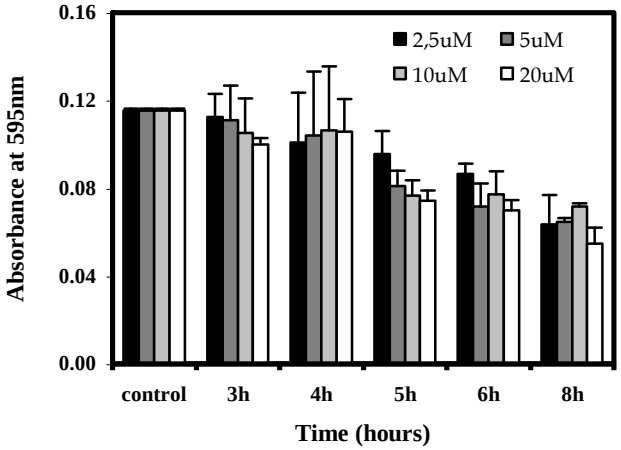

Figure 1. The viability of Huh 7 cells exposure of $CdCl_2$, in different concentrations, for 3, 4, 5, 6 and 8 hours, respectively.

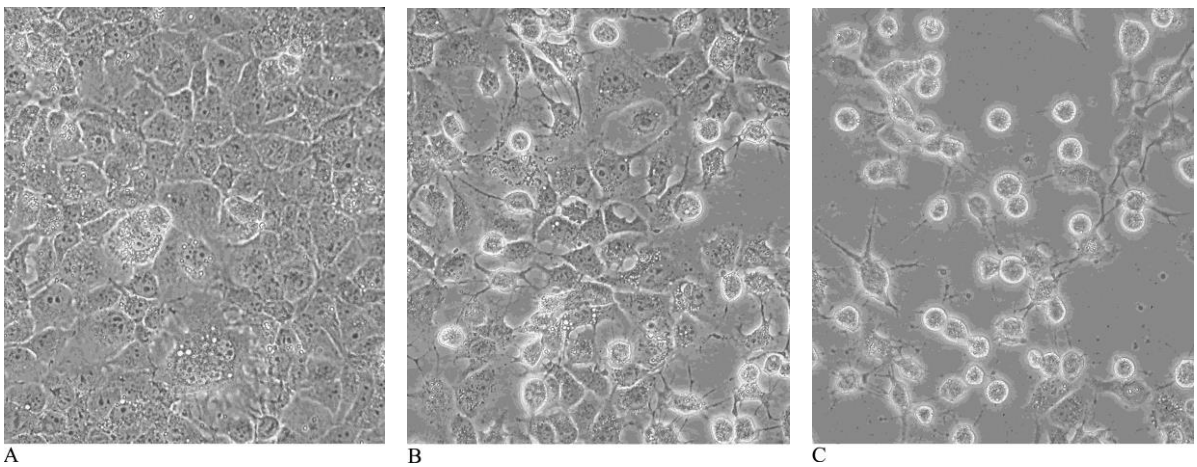

Figure 2. The effects of $CdCl_2$ on Huh 7 cells: control cells (A); cells treated 4 hours with 5µM $CdCl_2$ (B); cells treated 4 hours with 20µM $CdCl_2$ (C).

The phenotype of the Huh 7 cells treated with 5µM and 20µM $CdCl_2$ is shown in **figure2**. As it can be seen, the untreated hepatocytes (**Figure 2 - A**) present a uniform polygonal morphology generally being uninucleated, rarely binucleated, adherent to support

with clearly defined edges, shining uniformly distributed. After the treatment with 5μM $CdCl_2$ (**Figure 2 - B**) the cells suffered several morphological changes, losing the polygonal aspect and remaining connected through extensions similar to those of dendrites. The cell nuclei have a granular aspect and the cytoplasm is intensely vacuolated. The treatment with 20μM $CdCl_2$ (**Figure 2 - C)** determines the appearance of an important number of apoptotic cells, the hepatocytes phenotypic normal being rare. In this case, the intercellular connections are done through extension dendrites like, which are multiple physical connections with neighboring hepatocytes.

Figure 3-A shows the effect of Cd^{2+} administration on the CAT activity. The CAT activity increased by 42.2 and 34.2 % after 5 and 20 μM $CdCl_2$ treatment, respectively. The activity of GPX was up-regulated by 39.1% after 20 μM $CdCl_2$ administration (**Figure 3-B**).

The inner mitochondrial membrane is permeable to divalent cations, such as Ca^{2+}, Mn^{2+} and Cd^{2+}, due to a carrier for the cation electrical transport. During intoxication, Cd^{2+} is accumulated in the mitochondrial matrix probably generating a higher quantity of ROS, such as hydroxyl radical. Hydrogen peroxide is decomposed by CAT at high concentration, and by GPX at low concentration. GPX catalyses the glutathione-dependent reduction of hydroperoxides and hydrogen peroxide. The increases in CAT and GPX activity would indicate a higher concentration of hydrogen peroxide generated during the intoxication with Cd^{2+}. It has been demonstrated that Cd treatment produces ROS that cause cell damage [21]. Our results showed that endogenous antioxidant enzymes, such CAT and GPX, can neutralize cellular hydrogen peroxide produced after $CdCl_2$ intoxication.

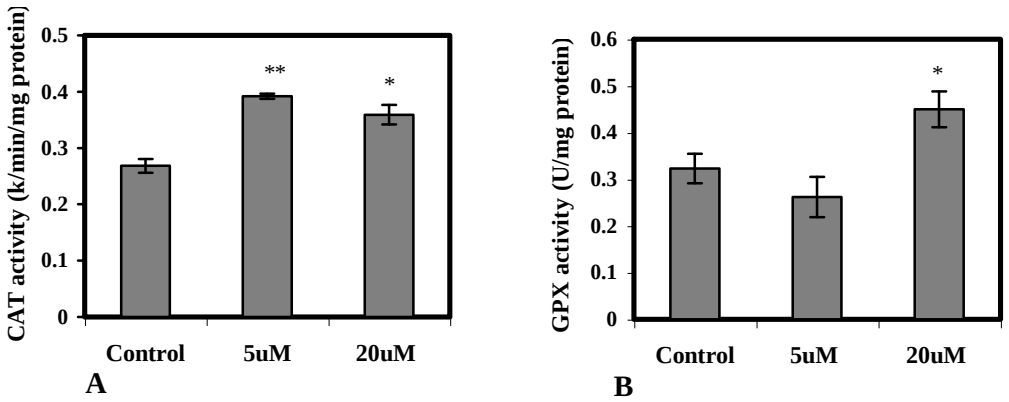

Figure 3. Variations in catalase activity (A) and GPX activity (B) in the Huh 7 cells treated 4 hours with 5μM and 20μM $CdCl_2$. Values are means ± SD. * P<0.05 vs. control; ** P<0.01vs. control.

The specific activity of G6PD was decreased by approximately 30%, for both Cd concentrations, possibly due to the generated oxygen reactive species (**Figure 4-A**). This enzyme catalyzes the oxidative branch of the pentose phosphate pathway, generating NADPH, an electron donor in reductive biosynthesis, which is used for GSH regeneration. This enzyme is also known as a sensitive target to oxidative stress [22]. G6PD was reported to be partially inhibited by cadmium in hepatocytes, but this enzyme was less sensitive than GR to Cd intoxication [23]. As a consequence the cellular NADPH content is diminished and the GR specific activity is decreased (**Figure 4-B**). The inhibition of this enzyme was directly dependent on the cadmium concentrations. At 5 μM $CdCl_2$ the GR activity was decreased by 29.5%, while, at 20 μM $CdCl_2$ the decrease was by 62%.

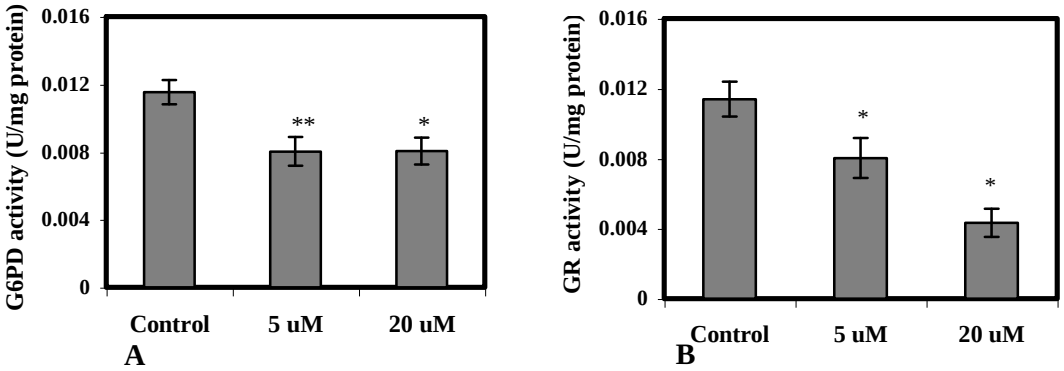

Figure 4. Variations in G6PD activity (A) and GR activity (B) in the Huh 7 cells treated with 5μM and 20μM $CdCl_2$ for 4 hours. Values are means ± SD. * P<0.05 vs. control; ** P<0.01vs. control.

Cd does not produce ROS through Fenton-type reaction and therefore cannot generate reactive oxygen species by itself [24]. However, Cd may interact with lipids and cause lipid peroxidation in tissues [25]. Alternatively, it replaces iron from cellular binding sites to increase the free iron level and lead to iron-induced oxidations (Fenton-type reaction) [26]. These factors may add synergistically to the elevation of cellular ROS [27]. To prevent Cd-induced damage, cells respond by turning on diverse signaling pathways and increasing the expression of genes that encode for stress-response/redox sensitive transcription factors, proteins such as metalloproteins, and antioxidant enzymes [28].

Conclusions

This study reveals that cadmium exerts an oxidative stress on Huh 7 cells. The treatment of Huh 7 cells for 4 hours with 5μM and 20μM $CdCl_2$ induced the elevation of the glutathione peroxidase and catalase activities, while, the specific activity of glucose-6-phosphate dehydrogenase and glutathione reductase was decreased. Our study showed that cadmium is able to induce oxidative stress in Huh 7 cells which is counteracted by a protective antioxidant mechanism.

References

1. JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES, *World Health Organ Tech.*, **930**, 30-41 (2006). BV
2. G. OBERDORSTER., *IARC Sci. Publ.*, **118**, 189-204 (1992).
3. M.H. BHATTACHARYYA, A.K. WILSON, S.S. TAJAN, M. JONAH., *Molecular Biology and Toxicology of Metals*, R.K ZALUPS, K. KORROPATRIC, eds., Taylor and Francis, London, 2000, pp. 34-74.
4. J. LIU, Y.LIU, S.M. HABEEBU, M.P. WAALKERS, C.D. KLAASEN, *Toxicology*, **147**, 157-166 (2000).
5. G.L DIAMOND, R.K. ZALUPS, *Toxicol. Pathol.*, **26**, 92-103 (1998).
6. C. C. BRIDGES, R. K. ZALUPS, *Toxicol Appl. Pharmacol.*, **204**, 274–308 (2005).
7. B. A. HART, C.H. LEE, G.S. SHUKLA, A. SHUKLA, M. OSIER, J.D. ENEMAN, J.F. CHIU, *Toxicology*, **133**, 43-58 (1999).
8. J. XU, D. MAKI, S.R. STAPLETON, (2003) *J. Biochem. Mol. Toxicol.*, **17**, 67–75 (2003).

9. A. GALAN, M.L. GARCIA-BERMEJO, A. TROYANO, N.E. VILABOA, E. BLAS, M.G. KAZANIETZ, *J. Biol. Chem.,* **275**, 11418–11424 (2000).
10. V. J. THANNICKAL, B.L. FANBURG, *Am. J. Physiol. Lung Cell Mol. Physiol.*, **279**, L1005–L1028. (2000).
11. H. SIES, *Oxidative stress. Oxidants and antioxidants*, H. SIES, ed., San Diego, CA: Academic Press, 1991, pp. XV-XXII.
12. D. ANDRES, M. CASCALES, *Biochem. Pharm.*, **64**, 267–276 (2002).
13. A.A. FRANCO, R.S. ODOM, T.A. RANDO, *Free Radical Biol. Med.*, **27**, 1122–1132 (1999).
14. E. CASALINO, G. CALZARETTI, C. SBLANO, C. LANDRISCINA, *Toxicology*, **179**, 37–50 (2002).
15. S.K. WATTANAPITAYAKUL, J.A. BAUER, *Pharmacol. Ther.,* **89**, 187–206 (2001).
16. H. AEBI, *Methods of Enzymatic Analysis*, H.U. BERGMAYER, ed., Chemie, 2nd edn., Vol.**2**, Weinheim, F.R.G., 1974, pp. 673-684
17. E. BEUTLER, *Manuel of Biochemical Methods*, Grune and Stratlon, Orlando, 1984, pp. 74-76
18. D.M. GOLDBERG, R.J. SPOONER, *Methods of Enzymatic Analysis*, H.U. Bergmayer H.U. ed., 3rd edn., Vol.**3**, Verlag Chemie, Dearfield Beach, 1983, pp. 258-265.
19. G.W. LÖHR, H.D. WALLER, *Methods of Enzymatic Analysis*, H.U. BERGMEYER, ed., Verlag Chemie Weinheim, Academic Press, Inc., New York, San Francisco, London, 1974, pp. 636-646.
20. O.H. LOWRY, N.J. ROSENBROUGH, A.L. FARR, B.J. RANDALL, (1951), .*J Biol Chem.*, **193**, 265-275 (1951).
21. T. ZHOU, G. ZHOU, W. SONG, N. EGUCHI, W. LU, E. LUNDIN, T. JIN, G. NORDBERG, *Toxicology*, **142**, 1-13 (1999).
22. K.L. MAIER, H. HINZE, B. MEYER, A.G. LENZ, *FEBS Letters*, **396**, 95-99 (1996).
23. L. MULLER, *Toxicol. Lett*., **30**, 259-265 (1986).
24. P. O'BRIEN, H.J. SALACINSKI, *Arch. Toxicol.*, **72,** 690–700 (1998).
25. D. MANCA, A.C. RICARD, H.V. TRA, G. CHEVALIER, *Arch. Toxicol.,* **68,** 364–369 (1998).
26. J.G. WARDESKA, B. VIGLIONE, N.D. CHASTEEN, *J. Biol. Chem.*, **261,** 6677–6683 (1986).
27. S.J. STOHS, D. BAGACHI,. *Free Rad. Biol. Med.,* **18**, 321-336 (1995).
28. J. CHIN-JU, R. HSIAO, S. STAPLETON, *J Biochem. Mol. Toxicol.*, **18**, 133-142 (2004)