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Role of Intracellular Ca^{2+}, Reactive Oxygen Species, Mitochondria Transmembrane Potential, and Antioxidant Enzymes in Heavy Metal-Induced Apoptosis in Fish Cells

L. X. Xiang, J. Z. Shao

College of Life Sciences, Zhejiang University, Hangzhou 310012, People's Republic of China

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Heavy metal pollution of waters is a major environmental problem. Most heavy metals are released into waters through anthropogenic activities such as industry and agriculture (Saygideger 2000). Fishes are known to absorb and accumulate heavy metals, including toxic metals such as Cd, Cr, Pb, As and Hg, which were not essential for their growth and development. However excess accumulation of these metals can be toxic to fishes. Indeed, the acute and chronic toxic effects of heavy metals have been studied in several fishes (Stabdi et al. 1999, Zsolt et al. 2001). However the cytotoxicity and subsequent molecular mechanisms remain unclear. Even in human and mammalian cell types, the mechanisms of heavy metals underlying the cytotoxicity are only defined as the role of inactivation of some essential enzymes through binding sulphydryl groups and production of Reactive Oxygen Species (ROS), the later of which is considered to be the first step in many toxic processes such as lipid oxidation, single strand-DNA damage and nitric oxide induction (Bayoumi et al. 2000). Little is known about the occurrence of apoptosis in heavy metal toxicity. Recently, we have used a grass carp (*Ctenopharyngodon idellus*) cell culture ZC7901 as an *in vitro* model system to study the cytotoxicity of heavy metals on fish cells, and the results showed that six heavy metals (Cd, Cr, Hg, Cu, As, Pb) employed in our experiments are all able to induce apoptosis in fish cells. It indicated that apoptosis is closely associated with the toxicity of heavy metals, so it is important to understand the mechanisms of heavy metal toxicity in fishes (Xiang et al. 2001).

Apoptosis is an active process in which cell death is initiated and completed in an orderly manner through activation and/or synthesis of gene products necessary for cell destruction (Green and Reed, 1998). It is regulated by a complicated mechanism. Although it was found that a number of chemical and biological stimuli can induce apoptosis in many cell types, the related molecular mechanism is not fully understood (Peter et al. 1997). Recently, it is generally believed that in certain cell systems, intracellular Ca^{2+}, ROS and mitochondrial transmembrane potential ($\Delta\Psi_m$) play an important role in the apoptotic process (Susin et al. 1999). However whether these factors are involved in fish cell apoptosis remain mainly unreported. The present study focuses on Cd^{2+}-induced apoptosis and the role of intracellular Ca^{2+}, ROS, $\Delta\Psi_m$ and antioxidant enzymes in this response to ascertain the mechanisms involved in the apoptotic toxicity of heavy metals in fish cells.

Correspondence to: J. Z. Shao

MATERIALS AND METHODS

The grass carp cell line, ZC-7901, derived from the carp lip epidermis was maintained in TC-199 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2m*M*-glutamine, 100U/mL of penicillin, 100μg/mL of streptomycin, and with 5.6% $NaHCO_3$ to pH7.0. Cells were routinely cultured twice a week at 27°C and maintained genetic homogeneity (2n=48).

The percentage of apoptosis was assayed according to the method described by Zhai et al (2000). Briefly, cells that had been incubated in the absence or presence of 1.8×10^{-5} m*M* Cd^{2+} for different times (0.5, 1, 2, 4, 6, 8 and 10 hr) were fixed with 70% ethanol for a night at 4°C, and washed by phosphate-buffered saline (PBS, 2.68 m*M* KCl, 1.47m*M* KH_2PO_4, 8m*M* Na_2HPO_4, 136.8m*M* NaCl) twice and suspended in 0.5mL of PBS, treated by final concentration 80μg/mL RNase I and stained with 50μg/mL propidium iodide (PI) for 30min at 4°C, then washed twice in PBS and harvested for FACS (Becton Dickinson FACsort, USA) analysis. PI was excited at 288nm and detected at 530nm.

Cells for apoptosis morphologic analysis were harvested at the end of treatment, washed twice in PBS and stained with 0.01% acridine orange (Sigma Chemical Co.) for 5min at room temperature. After being air-dried, the stained cells were observed under a Nikon fluorescent microscope at 510nm. The cell images were captured with a Nikon UFX-IIA color camera and printed with a Olympus digital–color printer P-330NE.

DNA ladder production was analyzed by gel electrophoresis as previously described (Xiang et al. 2001). In brief, 1×10^6 cells were harvested and washed twice in PBS after exposed to Cd^{2+} for 10 hr, and lysed with 1%SDS for 0.5hr at 37°C, then with 0.2g/L proteinase K (pH8.0) for 2hr. The lysate was extracted with phenol and phenol/chloroform (1:1), and the DNA was precipitated with ethanol overnight at -20°C; the DNA sample was applied on 1.5% agarose gel and visualized by staining with 30ng/mL ethidium bromide (Sigma Chemical Co.).

Intracellular Ca^{2+} levels in both control and Cd^{2+} treated cells were examined by previously described methods (Greagh et al. 2000). Briefly, the control cells and cells treated with Cd^{2+} at specified time intervals were stained with Fluo3-AM (Sigma Chemical Co.) at final concentration of 5μg/ml for 40 min at 37°C, then washed twice in cold PBS and harvested for FACS analysis.

Cytofluorometric determination of mitochondrial transmembrance potential ($\Delta\Psi_m$) in both control and Cd^{2+} treated cells were performed as described in literature (Del et al. 1991). Briefly, the control cells and cells treated with Cd^{2+} at specified time intervals were stained with Rhodamin 123 (Rh123, Sigma Chemical Co.) at final concentration of 0.5μg/mL for 20 min at 37°C, then washed twice in PBS and harvested for FACS analysis.

Intracellular superoxide anion (O^-_2) assay was performed as described by Enane (1993). For this, the cells that had been incubated in the absence or presence of

Cd^{2+} for different times were plated in a 96-well plate at a density of 1×10^5 cells/well, 100μL nitroblue tetrazolium (NBT, Serva Chemical Co., dissolved in PBS at 1mg/mL) was added into the cell culture for 1hr at 27°C, then the cells were fixed with methanol before washed twice in PBS. After the cells were centrifuged at 3000rpm for 5min, 20μL 2 *M* KOH and 140μL DMSO were added to the cells. The cells were read at 630nm in a microplate reader.

Intracellular hydrogen peroxide (H_2O_2) was measured followed the method described by Enane (1993). For this, 1×10^5 control and Cd^{2+} treated cells/well were mixed with 100μL PRS buffer (140m*M*NaCl, 10m*M* K_2HPO_4-KH_2PO_4 buffer pH7.0, 5.5m*M* Glucose, 0.56m*M* phenol red and 100μg/mL horseradish peroxidase), and maintained for 1hr at 27°C; then 10μl 1*M* NaOH was added to stop reaction, and the cells were read at 630nm in a microplate reader.

For total superoxide dismutase (SOD) activity analysis, 100μL cell extracts derived from control and Cd^{2+} treated cultures were mixed with 4.5mL of 50mM Tris-HCl buffer (pH 8.2), after 20 min incubation at 25°C, 100μL of 45mM pyrogallic acid methanol (PAM) was added. The rate of absorbance change at 325nm was monitored for 30 sec. in a 722-vis spectrophotometer. One unit of SOD is the enzyme able to prevent PAM from self-oxidation to 50% per min per mg of soluble protein (Yu et al. 2000).

For catalase (CAT) activity analysis, Cd^{2+} treated cultures were added to 1mL of 0.3% guaiacol phenol red; and 100μL cell extracts derived from control, after 5min incubation at 20°C, 250μL of 0.1*M* H_2O_2 was added and maintained for 15min, and then 0.5mL acetone was added. The rate of absorbance changes at 475nm in a 722-vis spectrophotometer. One unit of CAT is the amount of enzyme able to reduce H_2O_2 per min per mg of soluble protein (Yu et al. 2000).

Data from at least 5 experiments were expressed as mean±S.D and the groups compared statistically with Student's *t*-test for paired samples. A p value of < 0.05 or <0.01 was considered significant.

RESULTS AND DISCUSSION

We have previously characterized the dose- and time-dependent toxicity of Cd^{2+} in ZC7901 cell line. The TD_{50} (12 hr) value of Cd^{2+} on this cell line was found to be 1.8×10^{-5} *M*. Cells treated at this concentration, apoptosis typically occurred (Xiang et al. 2001), thus the concentration of 1.8×10^{-5} mol/L Cd^{2+} was used for all the experiments in present study. Here the kinetics of ZC7901 cell apoptosis in response to Cd^{2+} were further analyzed for varying time periods. Apoptosis was quantified by the assessment of DNA integrity through fluorescence-activated cell sorting after propidium iodide staining. The percentage of apoptotic cells in control cultures (without treated with $Cd2^{+}$) was 0.5%. The percentage of apoptotic cells after 0.5 hr of incubation with Cd^{2+} was unchanged from that of control. By 1 hr, the percentage of apoptotic cells was 5%, which increased to 15% by 2 hr. Following an 4-hr incubation with Cd^{2+} the percentage of apoptotic cells had increased to 46% and by 6 hr was 62%. After 8-hr and 10-hr treatment

with Cd^{2+}, approximately 74% and 82% of the cells were apoptotic by FACS analysis (Fig.1). In cells exposed to Cd^{2+} for 10 hr, evidence of apoptotic features, including changes in nuclear morphology, DNA fragmentation and formation of DNA ladder in agarose gel electrophoresis were observed. In contrast, cells untreated with Cd^{2+} appeared morphologically normal (Fig.2~3).

Intracellular free Ca^{2+} is thought to act as an important 'second messenger' in a variety of cellular signaling pathways and metabolic processes. Recent studies also showed that it plays a crucial role in the apoptotic process induced by many chemical stimuli such as dexamethasone and hydrogen peroxide (Lu et al. 1999). To examine whether intracellular Ca^{2+} is involved in Cd^{2+}-induced apoptosis in fish cells, we have evaluated the intracellular Ca^{2+} levels following the incubation of cells with 1.8×10^{-5} mol/L Cd^{2+} for varying time intervals by the specific intracellular Ca^{2+} fluorescent probe Fluo3-AM. As shown in Fig.4, incubation of cells with Cd^{2+} resulted in a rapid and significant ($P<0.01$) elevation in intracellular Ca^{2+}, reaching a mean maximum value after 0.5 hr of exposure. At this time point, an approximately 6-fold increase in Ca^{2+} was observed in comparison to that of control cells. After the 0.5 hr transiently elevation, Ca^{2+} levels subsequently tapered off to a relatively constant level which was about 1.7-fold higher than that of normal cells after 2 hours, and the Cd^{2+} treated cells seemed to maintain this slightly elevated Ca^{2+} levels throughout the apoptotic processes. This result demonstrated that increasing in cytosolic Ca^{2+} not only seems to be an early apoptotic signal mediator that play a fundamental role in initiating the final common pathways for the death program, but also plays a role in continuing the apoptotic processes.

The reactive oxygen species (ROS) are known to modulate a variety of cell functions, and they are considered to play a major role in heavy metal-induced carcinogenesis (Ye et al. 1999). Moreover, recent research has also implicated ROS as central mediators in apoptosis induced by many diverse cytotoxic stimuli (Zhai et al. 2000). To define the role of ROS in Cd^{2+}-induced apoptosis, we examined O_2^- and H_2O_2 generation in the Cd^{2+}-treated cells. Kinetic changes in O_2^- and H_2O_2 levels in response to treatment with Cd^{2+} are shown in Fig.5 (a, b). A dramatic and sustained elevation in O_2^- and H_2O_2 was observed respectively over the course of the experiment. Significant ($P<0.05$) increases both in O_2^- and H_2O_2 were typically noticed between control and experimental cultures as early as 0.5 hr post-incubation. By 1 hr, the levels of O_2^- and H_2O_2 reached their peaks followed by an elevated and progressively decaying plateau. As compared to controls, a 4-fold significant ($P<0.01$) elevation in O_2^- and H_2O_2 levels following a 1hr incubation with Cd^{2+} was observed, while the plateau phase of O_2^- and H_2O_2 contents was about 3-fold higher compared to the control cells during the entire 8-hr incubation period. The results from our experiments were consistent with earlier observations in the literature that in many cases, apoptosis is accompanied by an elevation of the cellular levels of reactive oxygen intermediates, and intracellular Ca^{2+} mediated the reactive oxygen species production and subsequent oxidant-induced activation of redox-sensitive transcription factors, phosphatases, tyrosine kinases and opening of permeable transition pores in mitochondria, which initiates the loss of $\Delta\Psi_m$, the later of which was a key event in the apoptotic

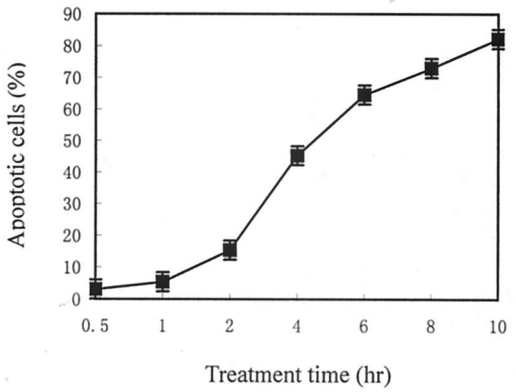

Figure 1. The kinetics of ZC7901 cell apoptosis in response to Cd^{2+}.

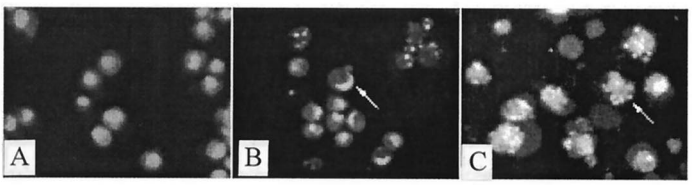

Figure 2. Fluorescence image of cells treated in the absence and presence of Cd^{2+}. A. Control cells; B. Cells treated with Cd^{2+} showing irregular chromatin condensation into crecentiform (↑ indicated, 200×) ; C. The chromatin became "pulverized" and granular (↑ indicated, 400×)

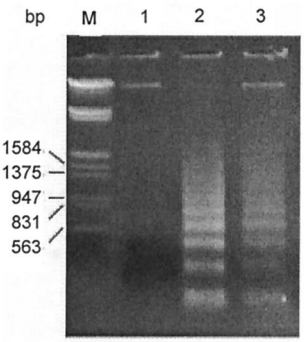

Figure 3. DNA ladder induced by Cd^{2+} in ZC7901 cells.
M: molecular mass markers (λ DNA digested by *Eco*RI and HidIII)
1: DNA of control cells
2~3: DNA ladders of cells induced by Cd^{2+}

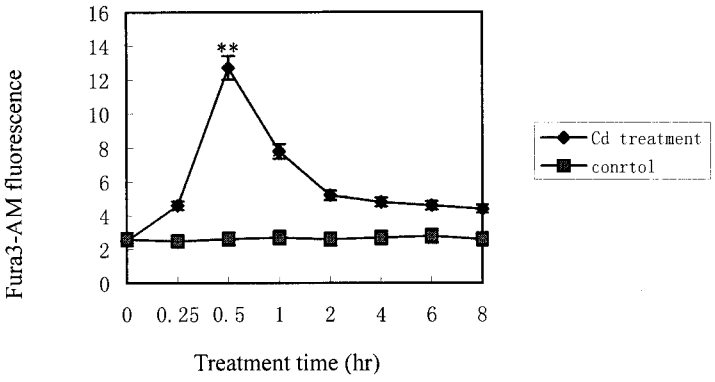

Figure 4. Changes of intracellular Ca^{2+} after Cd^{2+} treatment
Mean±SD, n=5; *indicates a significant difference from control (P<0.05), **indicates P<0.01

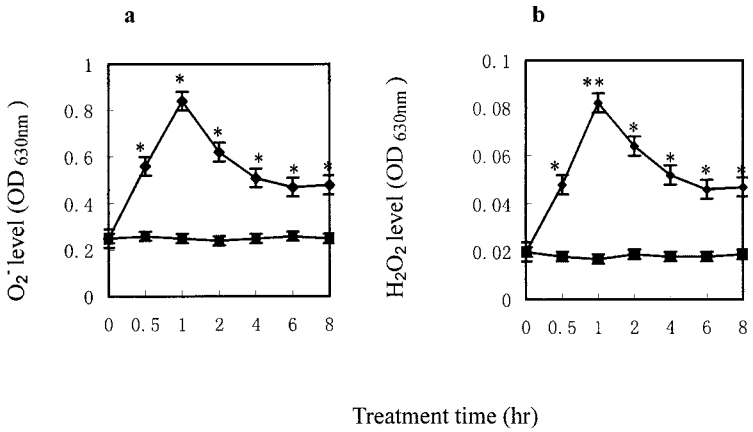

Figure 5 a. Changes of O_2^- levels; **b.** Changes of H_2O_2 levels
Mean±SD, n=5; *indicates a significant difference from control (P<0.05), **indicates P<0.01

pathway (Mignotte et al. 1998; Chakraborti et al. 1999). As a result, our study indicated that Ca^{2+} changes are probably involved in the induction of Cd^{2+}-mediated apoptosis, and ROS production is associated with the mechanisms of apoptotic damage.

Mitochondria changes are considered to be the key event in the apoptosis pathway in many experimental systems, including the enhanced production of oxygen radicals and loss of $\Delta\Psi_m$. Loss of $\Delta\Psi_m$, an established indicator of mitochondrial damage in the progression of apoptosis, was modulated by the elevated intracellular Ca^{2+} and ROS. These factors cause opening of permeable transition pores (PTP), the release of mitochondria contents, and caspase activation. In most models of apoptosis, caspases are a family of cysteine proteases that have become

recognized as key components of the apoptotic machinery (Marchetti *et al.* 1996; Kroemer *et al.* 1997). In order to investigate the role of $\Delta\Psi_m$ in Cd^{2+}-induced apoptosis, the mitochondria-specific dye Rh123 was employed for this purpose, and FACS analysis was conducted. Rh123 is a cationic fluorochrome that is specifically retained in the mitochondria of live cells due to the electronegativity of the inner membrane. Cell death or changes in mitochondria leading to loss of $\Delta\Psi_m$ result in the inability of cells to retain this dye, so the accumulation of Rh123 in cells is a reflection of $\Delta\Psi_m$. Kinetic data related to $\Delta\Psi_m$ are presented in Fig.6. The result showed that a time-dependent reduction in $\Delta\Psi_m$ was observed in Cd^{2+} treated cells as compared to controls during the incubation period. Within 1 hr of treatment, $\Delta\Psi m$ started to decreased and mitochondria damage occurred. However, the earliest time for $\Delta\Psi_m$ reduction to be significantly detectable ($P<0.01$) was 2 hr after the treatment. At this time, an approximately 50% decrease in $\Delta\Psi_m$ was seen as compared to control cells. The $\Delta\Psi_m$ decreased significantly thereafter in all the times tested. In particular, at 8-hr treatment with Cd^{2+}, cells almost completely lost their $\Delta\Psi_m$. Based on our data, we found marked changes in intracellular Ca^{2+} and ROS that precede $\Delta\Psi_m$. The intracellular Ca^{2+} and ROS generation occurred within the early stage (0-1hr) of apoptosis, whereas decreases in $\Delta\Psi_m$ took place at the later stages (2-8h). It appears that drop in $\Delta\Psi_m$ is a slightly later event in Cd^{2+}-induced apoptosis. The $\Delta\Psi_m$ may be a downstream mediator which contributes to the later stages of apoptosis and is responsible for the enhancement of Cd^{2+}-induced apoptosis at this stage.

Superoxide dismutase (SOD) and catalase (CAT) are major antioxidant enzymes responsible for scavenging the ROS generated via different mechanisms in cells (Yu *et al.* 2000). Under normal physiological conditions, ROS are kept in control by these enzymes. In other words, ROS are normal by-products of cellular metabolism and are detoxified by the antioxidant capacity of the cell. This delicate balance between the enzyme systems that produce ROS and the antioxidant enzymes that detoxify ROS is critical for normal structure and function of the cell. To understand the role of antioxidant enzymatic defenses against ROS injury following Cd^{2+} exposure, we examined the activities of SOD CAT in cells exposed to Cd^{2+} for various time periods. As shown in Fig.7 (a,b), no significant changes in the activities of both antioxidant enzymes were noted to occur between control and experimental cultures before 1 hr incubation. However, after 2 hr incubation, a time-dependently significant ($P<0.01$) decrease in the activities of both SOD and CAT was observed. By 4 hr, an approximately 1.5-fold decrease in SOD and CAT activities were present in cells treated with Cd^{2+}. By 6 hr, a greater than 2-fold decrease in SOD and CAT activities were present in treated cells. At the end of Cd^{2+} treatment, the activities of SOD and CAT in treated cells were 16% and 25% of the control respectively. These results suggested that Cd^{2+}-induced oxidative stress results in down regulation of the activities of SOD and CAT. Inhibition of these antioxidant enzymes would lead to the excessive production of ROS, which may overwhelm the antioxidant capacity of the cell. Thus, a balance between intercellular oxidants and antioxidants is perturbed, and the cells would undergo apoptosis. As a result, loss of SOD and CAT may be responsible for Cd^{2+}-induced apoptotic toxicity to cells.

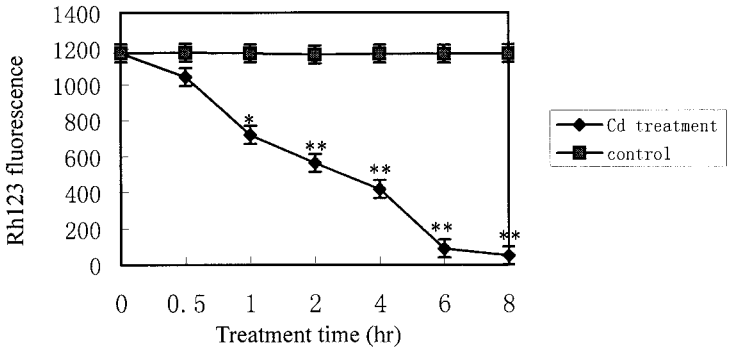

Figure 6. Changes of mitochondrial transmembrance potential ($\Delta\Psi_m$) during apoptosis process.
Mean±SD, n=5; * indicates a significant decrease from control (P<0.05),
** indicates P<0.01

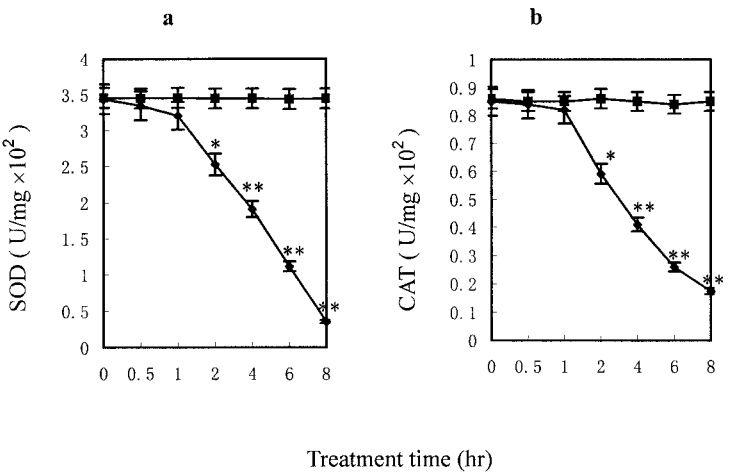

Figure 7a. Changes of SOD levels after Cd^{2+} treatment; **7b.** Changes of CAT levels after Cd^{2+} treatment.
Mean±SD, n=5; *indicates a significant difference from control (P<0.05), **indicates P<0.01

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