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Cadmium supplement triggers endoplasmic reticulum stress response and cytotoxicity in primary chicken hepatocytes

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ABSTRACT

Cadmium (Cd), a potent hepatotoxin, has been reported to induce endoplasmic reticulum (ER) stress in various cell types. However, whether such effect exists in bird is still unclear. To delineate the effects of Cd exposure on ER stress response, we examined the expression of 78-kDa glucose-regulated protein (GRP78) and alteration in calcium homeostasis in primary chicken hepatocytes treated with 2–22 μM Cd for 24 h. A significant decrease of cell viability was observed in chicken hepatocytes following Cd administration. In cells treated with Cd, GRP78 protein levels increased in a dose-dependent manner. In addition, GRP78 and GRP94 mRNA levels were elevated in response to Cd exposure. The increase of the intracellular Ca^{2+} concentration in chicken hepatocytes was found during Cd exposure. Cd significantly decreased the CaM mRNA levels in hepatocytes. These results show that Cd regulates the expression of GRP78 and calcium homeostasis in chicken hepatocytes, suggesting that ER stress induced by Cd plays an important role in the mechanisms of Cd cytotoxicity to the bird hepatocytes.

1. Introduction

Cadmium (Cd) is one of the most toxic pollutants of the natural and occupational environment. It is a relatively rare metal in the Earth's crust and does not have any positive biological function in organisms (Berg et al., 2008). Toxicity of this heavy metal for human and experimental animals is well known and widely reported (Järup and Akesson, 2009). Since Cd has a very long biological life which is estimated over 20 years, the possibility of this non-essential toxic metal bioaccumulating inside the body is inevitable. Whatever the route of exposure, the liver represents an important organ for the initial accumulation of Cd in the body and is potentially susceptible to Cd-induced cellular toxicities (Marettová et al., 2011). Both morphological and biochemical lesions of hepatocytes have been reported in animals exposed to Cd. Exposure of hepatocytes to Cd is known to up-regulate the expression of a number of stress proteins and results in activation of apoptotic pathways and consequently cellular damage. Cd produces hepatocytes injury through the generation of ROS and lipid peroxidation, which in turn depresses the hepatic functions (Amara et al., 2006; Lasfer et al., 2008).

Cd toxicity has also been linked to declines in body condition in birds (Kant et al., 2011; Pollock and Machin, 2009; Anteau et al., 2007). Several reports have been published on metals that induce toxicity in birds, altering their reproductive success, behavior, immune response, and biochemical processes (Binkowski et al., 2013). In some studied birds the thresholds of toxicity for Cd in liver was exceeded and lead some histological alterations: circulatory disturbances, retrogressive changes, inflammations and leukocytic infiltration (Binkowski et al., 2013). Investigations by our lab have found that dietary exposure to Cd caused histopathological changes, oxidative stress, endocrine disorder and apoptosis in cock testes (Li et al., 2010a). Cd inhibits the viability of the chicken splenic lymphocyte and induces the oxidative stress and subsequently DNA damage and apoptosis (Li et al., 2010b). Although there are several deleterious effects of Cd exposure on the liver in humans and rodents, there are no studies focusing on the hepatotoxicity in birds. Also the exact mechanism of toxic effects in birds is not clear.

These recent data suggest that ER could be a target of Cd toxicity. In mammalian cells, Cd has been shown to induce the typical markers of endoplasmic reticulum (ER) stress (Hirano et al., 1991; Liu et al., 2006; Hiramatsu et al., 2007; Biagioli et al., 2008). Cd or calcium ionophores which release calcium from the ER, inhibits normal protein synthesis and processing in the ER and cause ER stress (Li et al., 2000; Oğuz et al., 2007). The changes in Ca^{2+} concentration in cytoplasm as well as in different intracellular organelles could be responsible for induction of ER stress (Ferrari et al., 2002; Chang et al., 2011). CaM is a highly conserved Ca^{2+} binding protein that transduces Ca^{2+} signals into downstream effects influencing a range of cellular processes (Gaertner et al., 2004), including Ca^{2+} homeostasis. ER is well characterized as a mobilizable

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calcium store that sequesters excess cytosolic calcium and a reservoir for calcium signaling to maintain intracellular Ca^{2+} homeostasis (Xu et al., 2005). Various cell stress stimuli including growth factor withdrawal (Jayadev et al., 2000), hormonal stimulation (Wang et al., 1999), and toxicity treatment (Tagliarino et al., 2001) are known to alter the concentration of Ca^{2+} in the cytosol and the storage of Ca^{2+} in the intracellular organelles, which plays some roles in the cell stress pathway. As shown in a variety of cell types, Cd inhibits CaM activity, stimulates Ca^{2+}-activated depletion of internal ER Ca^{2+} stores (Rizzuto et al., 1998).

Cd is a very toxic metal that is described to cause multiple deleterious effects at different cellular levels. The hepatocytes were one of the main target cells for cadmium damage and toxicity produce. Cd has been shown to induce ER Stress in vivo and in vitro, so Cd-mediated toxicity is thought to involve the induction of ER Stress. However, susceptibility to Cd exposure in chicken hepatocytes is more significant than mammalian. To delineate the cytotoxicity of Cd on the bird hepatocytes in vitro, in this study, primary chicken hepatocytes were exposed to Cd. we investigated the effects of Cd exposure on ER stress response, we examined the expression of 78-kDa glucose-regulated protein (GRP78) and alteration in calcium homeostasis in primary chicken hepatocytes after exposure to Cd, we evaluated ER stress and cell injury in chicken hepatocytes.

2. Materials and methods

2.1. Chicken hepatocyte isolation, cell culture and exposure conditions

Chicken hepatocytes were isolated from chicken weighing 1.5–2.0 kg by two-step cllagenase perfusion technique (Fraslin et al., 1992). After the chick was anesthetized by an pentobarbital sodium (50 mg/kg) and anticoagulated by an Heparin Sodium (1750 u/kg), the liver was perfused via portal vein with pre-warmed calcium free liver perfusion medium, then the inferior vena cava was cut to effuse the buffer liquid when the liver was engorged. At first, perfusion was maintained at 15 to 20 mL per minute with 200 ml of calcium free liver perfusion medium until the liver became blanch, then the liver became soft followed by100 mL digestion buffer with 0.05 mg/mL of collagenase type IV (Sigma) and 0.06 mg/L calcium chloride in calcium free liver perfusion medium. After the perfusion, cells were dispersed in Basic Medium (BM) that consisted of William's medium E, 2 mg/mL Bovine Serum Albumin, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were filtered through sterilized gauze of pore sizes 150 and 75 μm to eliminate cell aggregate, centrifuged at 40g for 5 min, and washed three times with basic hepatocyte medium. Then 4×10^6 cells were seeded onto 6-well culture plate and incubated in Adherent Medium (AM) that consisted of William's medium E, 20% fetal bovine serum, 10^{-6} mol/L dexamethasone, 100 U/mL penicillin, 100 mg/mL of streptomycin and 1 mg/mL bovine insulin in a humidified atmosphere with 5% CO_2 at 37 °C. Following a 4 h incubation, the cells were washed with prewarmed phosphate buffered saline (PBS) buffer and then replaced AM with Growth Medium (GM) that consisted of William's E medium, 10% fetal bovine serum, 10^{-6} mol/l dexamethasone, 100 U/mL penicillin, 100 mg/mL of streptomycin and 1 mg/ml bovine insulin for 20 h before being used for subsequent experiments. Cells were then wash with PBS and supplied with GM containing $CdCl_2$ (Sigma) for 24 h. The control group was incubated for 24 h without $CdCl_2$.

2.2. Measurement of cellular Cd content

After a 24 h incubation with Cd (2.5, 5.0, 10 and 20 μmol/L), Cd concentrations in cells were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500 cs-Octopole Reaction Cell, Agilent Technologies, USA) at the Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences. In brief, cell suspensions were digested with nitric acid using a hot plate. The digest samples (1 mL) were diluted with 9 mL of 1% nitric acid (v/v, $>18\ \Omega\ cm^2$) immediately before the assay. The solutions obtained were then quantified by ICP-MS using freshly made Cd standards on the day of analysis. Results were given as nanogram per microgram of soluble cell protein.

2.3. Cell viability assay

Cell viability was determined by a colorimetric assay basedon the ability of viable cells to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is a yellow tetrazolium salt which forms a blue formazan dye precipitate that can be extracted using organic solvent when it is reduced by the mitochondria of metabolically active cells. Twenty-hour-old hepatocytes, seeded at a density of 2×10^4cells/well on collagen coated 96-well plates, were exposed to various concentrations of $CdCl_2$ (2 μmol/L, 6 μmol/L, 10 μmol/L, 14 μmol/L, 18 μmol/L, 22 μmol/L) in the presence of 5% FBS. After 24 h incubation, cell viability was evaluated using the MTT reduction method. Briefly, cells were incubated with 0.5 mg/mL MTT for 4 h at 37 °C; the solution was then removed and formazan salts dissolved with dimethyl sulphoxide, and the absorbance at 570 nm of the resulting solution was measured on a microplate reader (Synergy HT Multimode Reader, BioTek Instruments, USA).

2.4. Measurement of $[Ca^{2+}]i$

Intracellular free ionized calcium concentrations ($[Ca^{2+}]i$) were measured using the fluorescent calcium indicator fluo-3 acetoxymethyl ester (fluo-3-AM). This dye freely permeates the surface membrane, following hydrolysis by intracellular esterases, is trapped in cells as fluo-3. Various concentrations of Cd^{2+} treated chicken hepatocytes were washed three times with ice-cold PBS, scraped off in ice-cold PBS and centrifuged. Cells were loaded with 5 μmol/L fluo-3 AM in PBS (1 mL) for 30 min at 37 °C in the dark. Cells were washed three times with ice-cold PBS, resuspended in PBS (1 mL) and kept at 37 °C in the dark until measurement. The cell suspension (900 μL) was added to a quartz cuvette holder and the fluorescence was measured using a fluorescence spectrophotometer. The excitation and emission wavelengths used were 506 and 526 nm, respectively. $[Ca^{2+}]i$ was calculated from the ratio of the fluorescence intensity by using the formula: $[Ca2^{+}]i\ (nmol/L)=Kd(F-Fmin)/(Fmax-F)$, where F was the fluorescence intensity with nothing treatment, $Fmax$ and $Fmin$ are the fluorescence intensity after treatment of cells with 0.1% (V/V) aqueous Triton X-100 and 5 mM EGTA, respectively, and $Kd=244$ nM.

2.5. Determination of GRP78, GRP94, and CaM mRNA levels by quantitative RT-PCR

Total RNA was isolated from the cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen, China). The dried RNA pellets were resuspended in 50 μL of diethyl-pyrocarbonate-treated water. The concentration and purity of the total RNA was determined spectrophotometrically at 260/280 nm. First-strand cDNA was synthesized from 2 μg of total RNA using oligo dT primers and Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, China). Synthesized cDNA was stored at −80 °C before use. Primer Premier Software (PREMIER Biosoft International, USA) was used to design specific primers for GRP78, GRP94, CaM and β-actin based on known chicken sequences (Table 1).

Table 1
Primers used for quantitative real-time PCR.

Target gene	GenBank Accession no	Primer	Sequence (5′–3′)	PCR fragment length (bp)
Chicken				
GRP78	NM_205491.1	Forward	5′-GAATCGGCTAACACCAGAGGA-3′	118
		Reverse	5′–CGCATAGCTCTCCAGCTCATT-3′	
GRP94	NM_204289.1	Forward	5′-CAAAGACATGCTGAGGCGAGT-3′	186
		Reverse	5′-TCCACCTTTGCATCCAGGTCA-3′	
CaM	NM_205005.1	Forward	5′-GATGGAGTTGGTAAAATGAGGGAA-3′	166
		Reverse	5′-ACGCACTGGAAAACTAGGGTCA-3′	
β-actin	L08165	Forward	5′-ACCGCAAATGCTTCTAAACC-3′	93
		Reverse	5′-CCAATCTCGTCTTGTTTTATGC-3′	