Toxicology Letters 205 (2011) 183–189

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

# Dihydrolipoamide dehydrogenase and cAMP are associated with cadmium-mediated Leydig cell damage

Qihao Zhang[a,b,1], Ping Zou[a,1], Haichao Zhan[c], Minjing Zhang[a], Lei Zhang[a], Ren-Shan Ge[c,d,*], Yadong Huang[a,b,**]

[a] National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou 510632, China
[b] Guangdong Provincial Key Laboratory of Bioengineering Medicine, Jinan University, Guangzhou 510632, China
[c] The 2nd Affiliated Hospital, Wenzhou Medical College, Wenzhou 325035, China
[d] Population Council, 1230 York Avenue, New York, NY 10065, United States

ARTICLE INFO

*Article history:*
Received 13 April 2011
Received in revised form 3 June 2011
Accepted 4 June 2011
Available online 12 June 2011

*Keywords:*
Cadmium
Leydig cell
Dihydrolipoamide dehydrogenase
cAMP

ABSTRACT

Cadmium (Cd) directly inhibits testosterone production in Leydig cells, but its mechanism is still unclear. To further explore the signaling pathway of Cd-mediated toxicity to Leydig cells, various concentrations of Cd were cultured with R2C cells for 24 h, and two-dimensional gel electrophoresis (2DE)-based proteomics profiling was used to analyze the change of protein expressions. Cd caused a concentration-dependent inhibition of cell viability with $IC_{25}$, $IC_{50}$ and $IC_{75}$ of $2.42 \times 10^{-5}$ M, $4.83 \times 10^{-5}$ M and $7.39 \times 10^{-5}$ M, respectively. Cd significantly reduced progesterone production and mitochondrial membrane potential ($\Delta\Psi_m$) in a concentration-dependent manner. 2DE-based proteomics showed 34 protein spots with altered expression by 2-folds or more, and dihydrolipoamide dehydrogenase (DLD) was the hub in the network of these altered proteins. Real-time polymerase chain reaction (PCR) and Western blotting showed that Cd downregulated the expression of DLD. Cd also decreased intracellular levels of cyclic adenosine monophosphate (cAMP). The results suggest that DLD and cAMP may be key elements related to Cd toxicity to Leydig cells.



## 1. Introduction

Cadmium (Cd), an environmental pollutant, has an especially long biological half-life of 20–40 years, resulting in a significant accumulation in human body throughout whole life. Cd occurs naturally with other heavy metals such as zinc and lead in sulfide ores, and is used for electric batteries, electronic components, and nuclear reactors. There is remarkably increase in Cd pollution due to mining, metallurgy, manufacturing nickel–Cd batteries and as pigments and plastic stabilizers (Bertin and Averbeck, 2006) as well as cigarette smoking (Adams et al., 2011).

Numerous studies revealed that the testis was also a sensitive target for cadmium toxicity. Exposure to cadmium induced germ cell apoptosis and decreased daily sperm production, which might account for the decline of male fertility (Meeker et al., 2008; Oliveira et al., 2009; Valko et al., 2006). Cd is also an endocrine-disrupting chemical, and has a great impact on many endocrine systems including Leydig cells in males (Iavicoli et al., 2009). The testosterone is produced by Leydig cells in response to the stimulation of gonadotropin luteinizing hormone (LH) (Haider, 2004). LH binds to the LH receptor in the Leydig cell, activating stimulatory G protein via GTP binding, thus stimulating adenylate cyclase to elevate intracellular cyclic adenosine monophosphate (cAMP) level. One of kinase A targeted proteins is steroidogenic acute regulatory protein (StAR), which transports cholesterol to the inner mitochondrial membrane (Epstein and Ormejohnson, 1991; Papadopoulos et al., 2006), where it is metabolized to pregnenolone by the cytochrome P450 enzyme cholesterol side chain cleavage enzyme (CYP11A1). Pregnenolone is converted to testosterone by endoplasmic reticulum steroidogenic enzymes (Midzak et al., 2009).

Cd has been shown to inhibit Leydig cell steroidogenesis (Yang et al., 2003). One of possible mechanisms of Cd-mediated inhibition of steroidogenesis is the reduction of StAR expression (Gunnarsson et al., 2004). However, the detailed mechanism is still unclear. The objective of the present study was to study the possible mechanism of Cd-mediated toxicity in Leydig cells. In this regard, a rat R2C Leydig tumor cell line is used to study the toxicity of Cd. R2C cells were derived from a Leydig cell tumor in the rat testis (Shin et al.,

* Corresponding author at: Population Council & Rockefeller University, 1230 York Avenue, New York, NY 10065, United States. Tel.: +1 212 3278761; fax: +1 212 3277678.
** Corresponding author at: National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou 510632, China. Tel.: +86 20 85561215; fax: +86 20 85561215.
*E-mail addresses:* rge@popcbr.rockefeller.edu (R.-S. Ge), tydhuang@jnu.edu.cn (Y. Huang).
[1] Authors contributed equally to this study.

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doi:10.1016/j.toxlet.2011.06.003

1968) and have high basal steroidogenic activity (Jo and Stocco, 2004).

## 2. Materials and methods

### 2.1. Cell culture

Rat Leydig tumor R2C cells (ATCC, Manassas, VA) were cultured in F12 medium (Gibco, USA), supplemented with sodium pyruvate, $NaHCO_3$, 15% horse serum (HS), 2.5% FBS, 1% penicillin/streptomycin mixture and maintained at 37 °C in a 5% $CO_2$ incubator.

### 2.2. Cytotoxicity assay

Cell viability was determined using CCK-8 dye (Beyotime Inst Biotech, China) according to manufacturer's instructions. Briefly, $1 \times 10^4$ cells per well were seeded in a 96-well flat-bottomed plate, cultured at 37 °C for 12 h, and then placed in serum-starved (1.5% HS, 0.25% fetal bovine serum, FBS) conditions for further 24 h. Subsequently, cells were treated with $CdCl_2$ (Sigma, St. Louis, MO, USA) at various concentrations ($0 \times 10^{-5}$ M, $1 \times 10^{-5}$ M, $2 \times 10^{-5}$ M, $3 \times 10^{-5}$ M, $4 \times 10^{-5}$ M, $5 \times 10^{-5}$ M and $6 \times 10^{-5}$ M), for 24 h. Then, 10 μl CCK-8 dye was add to each well, cells were incubated at 37 °C for 2 h, and the absorbance was determined at 450 nm using a microplate reader (Bio-Tek, Winooski, VT, US). The 25% ($IC_{25}$), 50% ($IC_{50}$) and 75% ($IC_{75}$) maximal inhibitory concentrations of Cd to reduce cell number after 24 h culture will be calculated.

### 2.3. Progesterone radioimmunoassay (RIA)

Progesterone is the major steroid produced by R2C cells. Therefore, the progesterone production by the cell after exposure to Cd was measured (Freeman, 1987). R2C cells were treated with various concentrations of Cd for 24 h. The culture media were removed and centrifuged at 400 × *g* for 5 min at 4 °C. The supernatants were stored at −20 °C for progesterone assay. Progesterone levels in the media were measured using a RIA kit (Beijing North Institute of Biological Technology, Beijing, China) according to the manufacturer's instruction.

### 2.4. Measurement of mitochondrial membrane potential

The disruption of the mitochondrial electron-transport chain, membrane potential $\Delta\Psi_m$, or ATP synthesis has been associated with reduced Leydig cell steroidogenesis. Mitochondrial membrane potential was measured to evaluate the damage of mitochondrion induced by Cd. The fluorescent, lipophilic and cationic probe JC-1 (Beyotime, China) was employed to measure the mitochondrial membrane potential ($\Delta\Psi_m$) of R2C cells according to the manufacturer's instruction. Briefly, after different treatments, cells were cultured in 24-well plates and incubated with JC-1 staining solution (5 μg/mL) for 20 min at 37 °C. Cells were then rinsed twice with JC-1 staining buffer. Fluorescence intensity of both mitochondrial JC-1 monomers ($\lambda_{ex}$ 514 nm, $\lambda_{em}$ 529 nm) and aggregates ($\lambda_{ex}$ 585 nm, $\lambda_{em}$ 590 nm) were detected using a monochromator microplate reader (Safire II, Tecan, Switzerland). The $\Delta\Psi_m$ of R2C cells in each treatment group was calculated as the fluorescence ratio of red (i.e. aggregates) to green (i.e. monomers).

### 2.5. Two-dimensional gel electrophoresis, image analysis and mass spectrometry peptide sequencing

Two-dimensional gel electrophoresis (2-DE) maps and protein identification by matrix assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS) were performed to find out the altered expression of proteins in cells. R2C cells treated with Cd at different concentrations for 24 h were harvested and lysed for 40 min in cold lysis buffer. After centrifugation at 16,000 × *g* for 40 min, the supernatants were harvested as the total cellular protein extracts. The protein concentrations were determined using Bradford method. 2-DE was carried out by following the procedures established in our previous experiments (Zhang et al., 2010). Selected protein spots were excised from gels and the gel pieces were rinsed twice with distilled water, and then destained in potassium ferricyanide and ammonium bicarbonate in pH 8.0. After dehydrating with acetonitrile and drying in a SpeedVac, the samples were rehydrated in a minimal volume of sequencing grade porcine trypsin (Promega, USA) solution and incubated at 37 °C overnight. The supernatants were transferred and gels were extracted with extraction buffer. Peptide extracts and supernatants were combined and dried completely in the SpeedVac centrifuge. Protein digestion extracts were resuspended with 0.1% trifluoroacetic acid and then mixed in 1:1 ratio with a matrix consisting of a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 50% acetonitrile and 1% trifluoroacetic acid. Aliquots (0.8 μl) were spotted onto stainless steel sample target plates.

Peptide mass spectra were obtained on a 4800-plus MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA). Data were acquired in positive MS reflector using a CalMix5 standard to calibrate the instrument (ABI4700 Calibration Mixture). Both the MS and MS/MS data were processed by using the GPS Explorer software (V3.6, Applied Biosystems), the obtained MS and MS/MS spectra per spot were combined and submitted to MASCOT search engine (V2.1, Matrix Science, U.K.) by GPS Explorer software. The following parameters were used in the database searching: IPI Human database (V3.36), taxonomy of *Homo sapiens* (human), trypsin of the digestion enzyme, one missed cleavage site, partial modification of cysteine carboamidomethylated and methionine oxidized. Known contaminant ions (keratins) were excluded. Totally 69,012 sequences and 29,002,682 residues in the database were actually searched. MASCOT protein scores (based on combined MS and MS/MS spectra) greater than 61 were considered statistically significant differences ($p \leq 0.05$). The individual MS/MS spectra with statistically significant (confidence interval > 95%) best ion scores (based on MS/MS spectra) were also accepted.

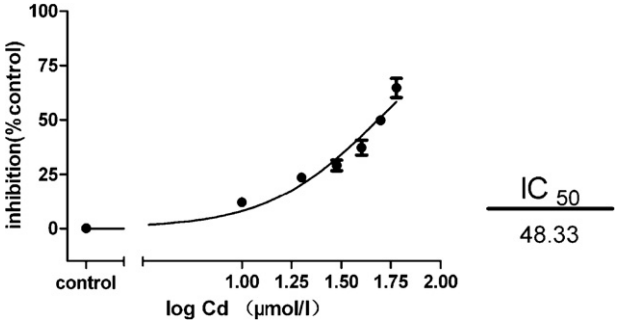


**Fig. 1.** Determination of inhibitory concentrations of cadmium on the viability of R2C cells. Cell viability was determined after exposure to various concentrations ($0–6 \times 10^{-5}$ M) of Cd. Cell viability was expressed as percentage relative to control. Mean ± SD, $n = 9$.

### 2.6. Real time PCR

Quantitative real-time polymerase chain reaction (PCR) was conducted to validate the altered expression of DLD gene (*Dld*). RNAs were extracted from cells using

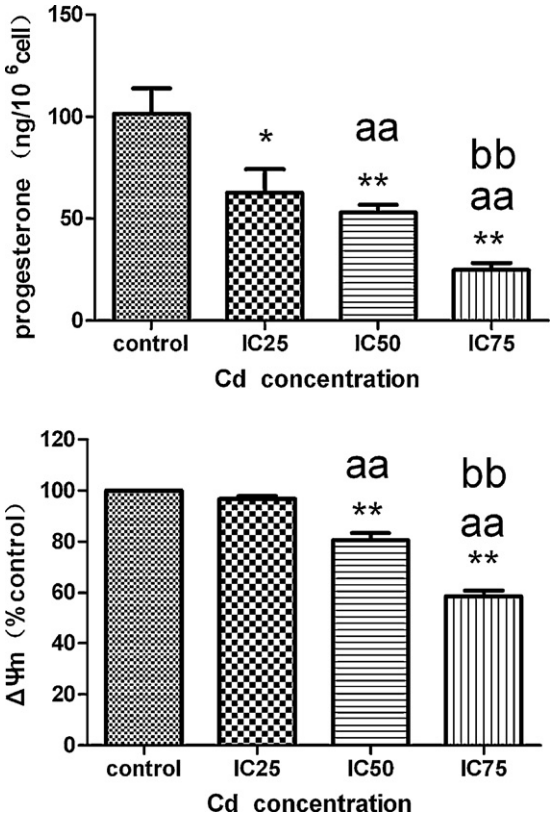


**Fig. 2.** Effects of cadmium on progesterone production and mitochondrial membrane potential of R2C cells. (A) Progesterone production (ng/$10^6$ cells). (B) Mitochondrial membrane potential ($\Delta\Psi_m$). Significant difference is designated at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control; $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$ compared to $IC_{25}$, $^{b}p < 0.05$, $^{bb}p < 0.01$, $^{bbb}p < 0.001$ compared to $IC_{50}$.