Cadmium up-regulates transcription of the steroidogenic acute regulatory protein (StAR) gene through phosphorylated CREB rather than SF-1 in K28 cells

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(Received September 9, 2014; Accepted December 25, 2014)

ABSTRACT — Cadmium is a widely used heavy metal in industry and affects the male reproductive system of animals, including humans, as a result of occupational and environmental exposures. However, the molecular mechanism underlying its effect on steroidogenesis in gonads remains unclear. In this study, we demonstrated that exposure of K28 mouse testicular Leydig tumor cells to cadmium led to a significant increase in the mRNA level, promoter activity and protein level of the steroidogenic acute regulatory protein (StAR), an essential factor for steroid biosynthesis. It has been well documented that StAR gene transcription is regulated by multiple transcription factors, including cAMP-responsive element binding protein (CREB) family members and SF-1. Cadmium treatment caused an increase in CREB phosphorylation but did not alter the CREB protein level in the nucleus. EMSA studies revealed that cadmium-induced phosphorylated CREB formed specific complexes with the proximal region of the StAR gene promoter. Furthermore, co-transfection with a CREB expression plasmid significantly increased cadmium-induced StAR promoter activity. However, the nuclear level and the affinity of SF-1 protein for the StAR proximal promoter were dramatically decreased upon exposure to cadmium. Taken together, these results suggest that cadmium up-regulates StAR gene expression through phosphorylated CREB rather than through SF-1 in mouse testicular Leydig cells.

Key words: Cadmium, Steroidogenic acute regulatory protein, Leydig cells, CREB, SF-1

INTRODUCTION

Cadmium is one of the most pervasive and persistent environmental contaminants, and it is found in soil, air and water. Humans and other mammals are exposed to cadmium in the environment through several routes, and the chance of exposure is rising accordingly with the increase in industrial usage of batteries, pigments, and electroplating equipment as well as several other applications. Because of its low excretion rate, cadmium has an extremely long biological half-life in the body, and it accumulates over time in the blood and in specific organs such as the liver and kidney as well as in the reproductive organs, including the placenta, ovaries and testes (Varga et al., 1993; Massanyi et al., 1995; Henson and Chedrese, 2004). The International Agency for Research on Cancer classified cadmium as a human carcinogen in 1993 (IARC, 1993), and numerous studies over several decades have provided evidence that cadmium is involved in carcinogenesis in diverse organs including the lung, liver, kidney and prostate (Waalkes, 2003; Goyer et al., 2004; Thompson and Bannigan, 2008).

The effects of cadmium on reproductive endocrinology have been reported; however, the results of these studies varied depending on the experimental models and dosages used. It was shown that cadmium interfered with normal gonadal function, as a significant reduction was observed in hormonal levels in vivo (Piasek and Laskey, 1994; Paksy et al., 1997; Piasek et al., 2002; Sen Gupta et al., 2004a) as well as in vitro (Mgbonyebi et al., 1998;
Cadmiun has been shown to enhance the expression of many classes of genes. It stimulates the expression of immediate early genes such as c-fos, c-jun and c-myc (Matsuoka and Call, 1995), apoptosis-inducing orphan nuclear transcription factors such as Nur77 (Shin et al., 2004), the tumor suppressor gene p53 (Zheng et al., 1996), genes encoding the protective molecules including metallothioneins (Imbert et al., 1990), glutathione (Hatcher et al., 1995) and stress-related (heat shock) proteins (Lee et al., 2002). The mechanisms responsible for cadmium-induced modulation of gene activity usually involve interference with cellular signaling at the levels of cell surface receptors, cellular calcium and zinc homeostasis, modulation of protein phosphorylation, and modification of transcription factors. However, the molecular mechanism underlying the effect of cadmium on steroidogenesis remains unclear, especially in testicular Leydig cells. In our present study, we found that cadmium activated StAR gene transcription in mouse testicular Leydig cells through phosphorylated CREB rather than SF-1.

**MATERIALS AND METHODS**

**Materials**

Cadmium chloride (CdCl₂) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and antibiotics were all obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). [α-³²P]-deoxy-CTP was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). Complete protease inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany). Polyclonal anti-StAR antibody was obtained from Affinity BioReagents (Golden, CO, USA), and anti-CREB antibody was supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-phosphorylated-CREB and anti-SF-1 antibodies were purchased from Upstate (Lake Placid, NY, USA). All other chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Co.

**Cell culture, treatments and viability analysis**

The K28 mouse testicular Leydig tumor cell line was a gift from Dr. C. Finanz (INSERM, CNRS, France). The K28 cells were maintained in DMEM containing 15% heat-inactivated FBS and antibiotics in humidified air containing 5% CO₂ at 37°C. The cells were treated with different doses of CdCl₂ or 0.5 mM cAMP in serum-free DMEM for different time periods as indicated in the Results section.

For the evaluation of cell viability, the cells were plat-
ed at concentration of 5 x 10^4 cells/well in a 24-well plate. After culture for 48 hr, the medium was replaced with serum-free medium followed by treatment with different doses of CdCl₂ for 8 hr. Then, the cells were washed with phosphate-buffered saline (PBS), and serum-free medium containing 1 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma) was added to each well. The cells were then incubated for a further 4 hr. The supernatants were carefully removed, 200 μL of dimethyl sulfoxide was added to each well, and the plate was agitated to dissolve the purple formazan product for 20 min. After complete solubilization, the absorbance values were measured at 562 nm using an Elx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Real-time PCR analysis

Total RNA was extracted from CdCl₂-treated K28 cells using Trizol reagent (Invitrogen). Briefly, after collecting the cells in Trizol reagent, the lysate was mixed with chloroform (5:1, v/v), shaken strongly for 30 sec and then centrifuged at 13,000 rpm for 15 min at 4°C. The RNA in the upper phase was precipitated by adding isopropropyl alcohol, washed once with 70% ethanol, and air-dried immediately. The RNA was then dissolved in water containing 0.1% diethyl pyrocarbonate (v/v). Reverse transcription was performed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). First, 2 μg of total RNA was denatured at 65°C for 10 min followed by incubation at 42°C for 1 hr in a 20 μL reaction mixture containing 1.0 U MMLV reverse transcriptase, 0.5 μg oligo (dT)₁₅ primer, 0.5 U/μL RNase inhibitor, and 1 mM dNTPs. The reverse-transcribed cDNA (1 μL) was used as a template for real-time PCR. PCR reactions were carried out using a real-time PCR machine (Rotor-Gene 3000; Corbett Research, Sydney, Australia) and Quantigene SYBR Green PCR Kits (Qiagen, Hilden, Germany) with mouse StAR primer pairs (forward: 5′-CCAGGAGCTGTCCTACATCCAG-3′; reverse: 5′-GTCGGAACACCTTGGCCACA-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer pairs (forward: 5′-AGAAAATCATCATTCTCATCCAG-3′; reverse: 5′-TTGTCATTGAGGCAATGCC-3′) as a quantitative control. Each 20 μL reaction mixture containing 1 μL of cDNA, 10 pM primers and 10 μL of reaction buffer was incubated for 3 min 95°C for denaturation, followed for 50 cycles of 95°C for 20 sec, 60°C for 10 sec, and 72°C for 20 sec. The fluorescence signals were analyzed with the Delta-Delta C, Reactive Quantitation method using Rotor-Gene 6 software.

Preparation of whole-cell and nuclear extracts (NEs)

Preparation of whole cell lysate was carried out as follows. Briefly, the cells from different treatment groups were washed two times with ice-cold PBS followed by scraping and collection by centrifugation. The pellets were lysed for 30 min in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.2% SDS, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM NaF, 1 mM Na₂VO₃, and protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation at 13,000 rpm for 15 min, the supernatant was assayed for whole-cell proteins.

NEs were prepared from cells as previously described (Dignam et al., 1983) with minor modifications. In brief, the cells were washed with PBS and collected by scraping. The cells were pelleted by centrifugation at 3,000 rpm for 5 min and submerged in ice-cold lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.5 mM KCl, 0.5 mM dithiothreitol, 1 mM PMSF, 5 mM NaF, 1 mM Na₂VO₃ and protease inhibitor cocktail on ice for 15 min. Subsequently, 0.1% Nonidet P-40 was added, the suspensions were gently mixed, and the nuclei were immediately collected by centrifugation at 4,000 rpm 10 min. The supernatant was discarded, and the crude nuclear pellet was washed twice with lysis buffer, omitting Nonidet P-40. The pellet was resuspended in extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.4 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 1 mM PMSF, 5 mM NaF, 1 mM Na₂VO₃, and protease inhibitor cocktail for 30 min in rotation at 4°C. After removing the debris by centrifugation at 13,000 rpm for 20 min, the supernatant was harvested and assayed for nuclear proteins. The protein concentrations of the cell extracts were determined using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting

Forty micrograms of whole-cell lysates and 60 μg of NEs were separated on 10% SDS-polyacrylamide gels after boiling for 5 min in Laemmli sample buffer. The proteins were then transferred to nitrocellulose membranes (Whatman, Dassel, Germany). The membranes were blocked overnight at 4°C in Tris-buffered saline containing 0.1% Tween-20 (TBS-T, pH 7.6) and 5% non-fat dry milk; they were then incubated with specific primary antibodies that recognize STAR, SF-1, CREB and phosphorylated CREB for 3 hr at room temperature. After incubation with primary antibodies, the membranes were washed three times with TBS-T, incubated with horserad-
ish peroxidase-conjugated secondary antibody against rabbit IgG for an additional 2 hr, and washed again. Immuno-reactive bands were visualized via chemiluminescence using Amersham ECL Western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, England) followed by exposure to X-ray film (Carestream Health Inc., Rochester, NY, USA). The level of phosphorylated or total CREB was assessed by stripping and reprobing using the same membrane. Horseradish peroxidase-conjugated anti-beta-actin antibody (Santa Cruz) was used as a loading control for whole-cell proteins.

**Transient transfection and luciferase activity assay**

The 5'-flanking regions of the rat StAR gene, -2200 StAR/Luc and -150 StAR/Luc (Lee et al., 1999), were cloned into the pGL3-Basic vector. The CREB expression construct was kindly provided by Dr. Hueng Sik Choi (Chonnam National University, Gwangju, Korea). For transient transfection, the K28 cells were split into 24-well plates at densities of 5 x 10⁴ cells/well one day prior to transfection. Transfection studies were conducted with Lipofectamine Plus reagent (Invitrogen) according to the instructions provided by the manufacturer. In brief, the cells in each well were transfected with 0.4 μg of StAR luciferase reporter constructs and 0.1 μg of pSV-β-galactosidase expression plasmid. The effects of CREB on cadmium signal transduction were tested in co-transfections with 0.4 μg of -2200 StAR/Luc and 0.4 μg of pcDNA-CREB expression constructs. The total amount of DNA used in the transfections was equalized with empty pcDNA3 vector. After incubation for 40 hr, the medium was replaced with serum-free medium followed by treatment with different doses for 8 hr. The cells were then harvested, and luciferase activity in the cell extracts was measured according to the standard method using a MicroLumat Plus LB 96 V (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was normalized to β-galactosidase activity to compensate for variations in transfection efficiency.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed as described by Manna et al. (2002, 2003). The double-stranded oligonucleotides with 5'-GG overhangs were end-labeled by filling in with [α-32P]-dCTP using Klenow enzyme (Roche) at 37°C for 45 min. The probes were purified using Probe-Quant G50 micro columns (Amersham Biosciences, Buckinghamshire, UK), and excess unincorporated [α-32P]-dCTP was removed. NE (10 μg) was pre-incubated in 10 mM HEPES (pH 7.9), 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol, 1 μg poly dL-dC, and 40 ng/μL BSA for 15 min at room temperature. Subsequently, 32P-labeled double-stranded DNA probe (greater than 6,000 cpm/μL) was added, and the mixtures were incubated for a further 15 min at room temperature. For the assay in which antibodies were used to block DNA-protein complex formation, NEs were incubated with a specific antibody against phosphorylated CREB, SF-1, for 1 hr on ice before the addition of the labeled probe. Non-immune rabbit IgG (Santa Cruz) was used as a negative control. When competitors were employed, a 50-fold molar excess of unlabeled oligonucleotide competitor was added to the reaction mixture containing NE and the labeled probe, and the mixture was incubated for 15 min. The DNA-protein complexes were then subjected to electrophoresis at 200 V for 90 min using a pre-cooled and pre-run native 5% polyacrylamide gel in 0.5 X TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0). The gel was vacuum-dried and exposed to X-ray film at -70°C overnight.

**Statistical analysis**

The data are expressed as the mean ± standard error (S.E.). Experimental data were analyzed using analysis of variance (ANOVA), and significant differences among the means were determined by Duncan’s multiple-range test at a 95% confidence level (SAS 9.2; SAS Institute, Cary, NC, USA).

**RESULTS**

Cadmium increases the levels of StAR mRNA and protein as well as StAR promoter activity

To evaluate the effect of cadmium on male reproductive systems, K28 mouse testicular Leydig tumor cells were incubated with various concentrations of cadmium for different time periods. After exposure to cadmium, real-time PCR analysis was performed to determine whether the StAR mRNA level was altered. As shown in Fig. 1A, cadmium significantly increased the StAR mRNA level in a time-dependent manner. The mRNA level began to increase after 1 hr and continued increasing up to 12 hr, after which it declined. To further explore
the induction pattern of StAR mRNA upon cadmium administration, cells were incubated with increasing doses of cadmium for 8 hr. Incubation with 5 μM cadmium slightly increased the StAR mRNA level, and it was elevated maximally at cadmium concentrations between 30 and 40 μM; subsequently, the StAR mRNA level declined (Fig. 1B). These results indicated that cadmium exposure induced StAR gene expression in both a dose- and time-dependent manner. Because treatment with 30 μM cadmium for 8 hr caused a maximal increase in the StAR mRNA level, this treatment condition was chosen for the subsequent experiments. To analyze the effect of cadmium on cell viability, K28 cells were incubated with increasing doses of cadmium for 8 hr, and cell survival was determined using the MTT assay. As shown in Fig. 1C, exposure of cells to up to 50 μM cadmium for 8 hr did not cause any significant change in viability. Treatment with 100 μM cadmium caused a slight reduction in viability (85% cell survival) compared with the untreated cells, which indicated that the cadmium treatment conditions (30 μM and 8 hr) chosen for this study have no effect on cell viability.

To confirm that the cadmium-induced up-regulation of StAR mRNA was attributable to the transcriptional activation of the gene, a luciferase activity assay using two constructs containing -2,200 bp and -150 bp regions of the StAR gene (Lee et al., 1999) was performed. The results showed that the luciferase activity of both reporter constructs was increased by cadmium concentrations of up to 20 μM compared to the basal level. A slight decrease was observed at 30 μM; however, the luciferase activity remained elevated over the basal level (Fig. 2A). In addition, immunoblotting was performed to determine whether cadmium also induced the expression of StAR protein. Similar to its effects on StAR mRNA expression, cadmium markedly increased StAR protein levels in a concentration-dependent manner. As shown in Fig. 2B, 30 μM cadmium induced a 6-fold increase in StAR protein. This result, together with that of real-time PCR analysis and the luciferase activity assay, demonstrates that cadmium induces StAR protein expression by transcriptional activation of the gene in mouse testicular Leydig cells.

Cadmium regulates the proteins involved in StAR gene transcription

To further examine the up-regulation of StAR gene expression by cadmium at the transcriptional level, we focused on SF-1 and CREB because they are both crucial transcriptional regulators of StAR gene expression in response to trophic hormones. K28 cells were incubated with increasing doses of cadmium for 8 hr, and then whole-cell lysates and NE were prepared for immunoblotting. The results shown in Fig. 3 indicated that both SF-1 and CREB proteins were maintained at a high level in K28 cells. The constitutive expression of SF-1 was considered decreased in a dose-dependent manner by cadmium administration, and SF-1 expression was inversely correlated with the StAR level (Fig. 2B). In contrast
Fig. 2. Effects of exposure to cadmium on promoter activity and protein expression of StAR in K28 cells. (A) Cells were transfected with 5'-deleted StAR promoter/luciferase constructs (-2200 StAR/Luc or -150 StAR/Luc) in the presence of β-galactosidase expression plasmids. After 40 hr of transfection, the cells were incubated with increasing doses of CdCl₂ (5, 10, 20, 30 μM) for a further 8 hr. Luciferase activity in the cell lysates was determined, and β-galactosidase activity was used as an internal control for transfection efficiency. The results are shown as the mean ± S.E. from three independent experiments performed in quadruplicate and are expressed as fold activation over cells transfected with the -150 StAR/Luc construct without cadmium treatment. (B) Cells were treated with increasing doses of CdCl₂ (5, 10, 20, 30 μM) for 8 hr. StAR protein in whole-cell lysates was determined by immunoblotting. Immunoblots of the upper panel are representative of at least five independent experiments. The amount of total protein loaded in each lane was determined by reprobing with anti-β-actin antibody. For both proteins, the densities of the bands were quantified in three independent experiments. The values were normalized to the corresponding β-actin band and expressed as fold induction compared with the untreated control (mean ± S.E.). * indicates \( p < 0.001 \).

Fig. 3. Effect of exposure to cadmium on the expression of proteins involved in steroidogenesis in K28 cells. Cells were treated with increasing doses of CdCl₂ (5, 10, 20, 30 μM) for 8 hr. (A) Whole-cell lysates were prepared, and the level of SF-1 was determined by immunoblotting. Equal loading was confirmed using β-actin. The experiment was repeated four times, and typical results are shown (mean ± S.E.). (B) Nuclear extracts were isolated as described in “Materials and methods”, and phosphorylated CREB was detected by immunoblotting. The membrane was stripped and reprobed with an anti-CREB antibody. The density of each band was quantified, and the ratios of phosphorylated CREB to CREB were calculated from three independent experiments (lower panel, mean ± S.E.). The results are expressed as fold induction compared with the untreated control. * indicates \( p < 0.001 \).
to the change in the SF-1 level, cadmium treatment did not significantly alter the nuclear CREB level. Interestingly however, cadmium induced the phosphorylation of CREB, as shown in Fig. 3B. The phosphorylated form of the protein was not detected in untreated cells, whereas a slight increase in phosphorylation occurred when the cells were treated with cadmium between 10 μM and 20 μM. This induction was remarkably increased upon treatment with 30 μM cadmium. Analysis of the phosphorylated CREB to CREB ratio revealed significant accumulation of phosphorylated CREB in cells treated with 30 μM cadmium, and these data demonstrate that activation of CREB by cadmium involved protein phosphorylation rather than protein synthesis.

**Cadmium increases binding of phosphorylated CREB, but not SF-1, to the STAR promoter**

The direct involvement of the transcription factors described above in cadmium-induced up-regulation of StAR was explored next. To determine whether phosphorylated CREB and SF-1 proteins bind to the StAR promoter and to investigate the contribution of cadmium to the binding of these proteins to the StAR promoter, EMSA was performed using nuclear proteins extracted from cadmium-treated K28 cells. Earlier studies have shown that the -150/-20 bp region was considerably conserved among the StAR promoters of different species including mice, rats, and humans, and this region had three 5’-canonical CRE half-site sequences and three putative SF-1 recognition motifs (Sugawara et al., 1997; Wooton-Kee and Clark, 2000; Manna et al., 2002, 2003). As shown in Fig. 4B, a 32P-labeled -96/-67 probe containing the three CRE half-sites (as depicted in Fig. 4A) formed specific binding complexes with NEs from K28 cells (lane 2), and the levels of these complexes were increased in NEs from cells treated with cadmium at 20 and 30 μM (compare lane 2 with lanes 5 and 6). The DNA-protein complexes were diminished following addition of a phosphorylated CREB antibody, as shown in the immunoblotting experiment (lane 11), compared to the addition of nonimmune

![Fig. 4. Binding of proteins present in cadmium-treated K28 NEs to the CRE and SF-1 regions. (A) Schematic diagram revealing the presence of three putative CRE half-sites and SF-1 binding sites in the mouse StAR promoter (-151 to -1 bp). The oligonucleotide probes used for EMSA are illustrated. K28 cells were treated for 8 hr with increasing doses of CdCl2 (5, 10, 20, 30 μM) and NEs were assessed in EMSA to analyze protein-DNA binding using a 32P-labeled CRE element (-96/-67 bp) (B) or SF-1 (-106/-92 bp) (C) as described in “Materials and methods”. Cold competitors were used at 50-fold molar excess. An antibody competition assay was carried out with specific antibodies against phosphorylated CREB and SF-1. Nonimmune IgG antibody was used as a negative control. The experiments were repeated three times, and representative results of one experiment are shown.](image-url)
IgG (lane 14). The specific complexes nearly disappeared after addition of a 50-fold molar excess of unlabeled oligonucleotides (lane 7). To identify the cadmium-responsive CRE half-site, a competition study was performed with cadmium-treated K28 NEs using oligonucleotides with mutations in each CRE half-site. The DNA-protein complexes competed with oligonucleotides with mutations in the CRE1 site (lane 8) and the CRE3 site (lane 10), while oligonucleotides with mutations in the CRE2 site were not able to compete with these complexes (lanes 9). The DNA-protein binding specificity was also assessed by competition with CRE2 and consensus CRE sequences (Manna et al., 2002). After the cold CRE2 was added, the amount of complex formed (lane 12) seemed to be decreased by about 50% compared to lane 6 and when the consensus CRE sequences added, the formation of the complex was greatly decreased (lane 13). These results suggest the critical involvement of the CRE2 site in DNA-protein complex formation, which is in agreement with previous findings demonstrating that, among the three CRE half-sites, CRE2 is the major site involved in StAR promoter activation (Manna et al., 2002, 2003).

The -150/-20 bp region of the StAR gene was shown to contain three putative SF-1 recognition motifs (Manna et al., 2003 and as illustrated Fig. 4A). Notably, the SF-1/3 region located between -106 and -92 bp upstream of the transcriptional start site was previously demonstrated to play a more important role in StAR promoter activation compared with the SF-1/1 and SF-1/2 sites (Manna et al., 2003). As indicated in Fig. 4C, the 32P-labeled SF-1/3 probe formed specific binding complexes with NEs of K28 cells (lane 2); however, the levels of these complexes were significantly decreased by cadmium in a dose-dependent manner (compare lanes 2 and 5). The binding was greatly diminished by addition of a 50-fold molar excess of unlabeled oligonucleotides (lane 6), and the specific antibody against SF-1 also markedly decreased DNA-protein complex formation (lane 7). The specificity of the complexes was further verified using a nonimmune IgG antibody that did not affect SF-1/3 site-NE binding (lane 8).

**Over-expression of CREB protein enhances cadmium-induced StAR promoter activity**

Additionally, we attempted to determine whether an increased level of phosphorylated-CREB can elevate StAR promoter activity. K28 cells were transfected with the -2200 bp StAR reporter construct in the absence or presence of CREB expression plasmids. The total amount of transfected DNA was kept constant using empty vector. After 40 hr of transfection, the cells were incubated with increasing doses of CdCl2 (5, 10, 20, 30 μM) for an additional 8 hr. Luciferase activity in the cell lysates of each group was determined, and β-galactosidase activity was used as an internal control for transfection efficiency. Luciferase activity values in untreated cells were set to 1.0, and the relative luciferase activity in CdCl2 treated cells was calculated (mean ± S.E.). The experiments were performed in duplicate and repeated three times. *indicates p < 0.001.

**Fig. 5.** Effects of exposure to cadmium on StAR promoter activity in K28 cells over-expressing CREB. Cells were transfected with a -2200 StAR promoter/luciferase construct in the presence of β-galactosidase expression plasmids with or without CREB expression plasmids. The total amount of transfected DNA was kept constant using empty vector. After 40 hr of transfection, the cells were incubated with increasing doses of CdCl2 (5, 10, 20, 30 μM) for an additional 8 hr. Luciferase activity in the cell lysates of each group was determined, and β-galactosidase activity was used as an internal control for transfection efficiency. Luciferase activity values in untreated cells were set to 1.0, and the relative luciferase activity in CdCl2 treated cells was calculated (mean ± S.E.). The experiments were performed in duplicate and repeated three times. *indicates p < 0.001.

**DISCUSSION**

The purpose of this study was to determine the effect of the heavy metal cadmium on StAR gene expression in steroidogenic cells. These results indicated that cadmium induced the transcription of the StAR gene in mouse testicular Leydig cells in both a time- and dose-dependent manner (Fig. 1). Cadmium was also shown to stimulate the activity of the StAR promoter (Fig. 2A), thus suggesting that this heavy metal induces the transcription of the StAR gene. Cadmium was previously reported to increase the intracellular cAMP level (Cook et al., 1985; Kumar and Bhattacharya, 2000) and was also demonstrated to bind to the CRE and promote transcriptional activity of stress genes in HepG2 cells (Tchounwou et al., 2001). Additionally, earlier reports suggested that cadmium can directly activate PKC (Long, 1997), and recent studies have dem-
Effects of cadmium on StAR expression

Demonstrated the possible role of PKC in StAR expression in mouse testicular Leydig cells (Jo et al., 2005; Manna et al., 2009). These results suggested that the cAMP/PKA and PKC pathways might be involved in the cadmium-induced transcriptional activation of the StAR gene in testicular Leydig cells. However, Gunnarsson et al. (2004) and Sen Gupta et al. (2004b) have reported that cadmium caused the inhibition of testosterone production via lowered expression of StAR in rat testicular Leydig cells in vivo. It might be possible that the effects of cadmium on male reproductive system would vary depending on the experimental systems. Thus we are currently conducting experiment on the levels of hormones as well as the proteins involved in hormone biosynthesis in cadmium-treated mouse Leydig cells in vivo in order to test whether or not cell line study is consistent with in vivo study.

The response to cAMP at the molecular level is typically mediated by a palindromic conserved sequence (5′-TGACGTCA-3′) commonly referred to as the cAMP response element (CRE) (Lalli and Sassone-Corsi, 1994). However, similar to cAMP-regulated steroid hydroxylase genes, the StAR gene lacks a consensus CRE (Waterman, 1994). Members of a large family of basic leucine zipper (bZIP) CRE binding factors, including the CRE binding protein (CREB), the CRE modulator protein (CREM), and the activating transcription factor (ATF-1), have all been shown to interact with this sequence. Members of the CRE binding factor family can serve as activators or repressors of transcription, and these proteins can homodimerize and heterodimerize via a specific interaction domain (Manna et al., 2002). Three genes, CREB, CREM and ATF-1, share a profound homology and constitute the CREB/CREM subfamily (CREB/CREM). These genes encode proteins that mediate transcriptional activation of the StAR gene (Manna et al., 2002). Previous reports have shown that 150 nucleotides from the transcriptional start site were critical for cAMP and trophic hormone-induced StAR gene activation (Manna et al., 2003) and the three CRE half sites (-96/-67 bp) have been characterized within the cAMP-responsive region (-151/-1 bp) of the mouse StAR gene (Manna et al., 2002). In this study, treatment of 10 mM cadmium induced 2-fold increase in the phosphorylated CREB protein, while the StAR protein level was increased 5-fold. The result could suggest that cadmium-induced StAR expression may be caused by more transcription factors including CREM and ATF-1, as well as CREB protein. Further study is required to identify all transcriptional factors participating in cadmium-induced StAR expression.

It had been reported that cadmium treatment induced the mitogenic signaling in the 1LN prostate cell line through the increase of transcription factors NFkB and CREB. (Misra et al., 2003). Also Kondo et al. (2012) demonstrated that treatment of cadmium activate extracellular signal-regulated kinase 5 (ERK5) which is a member of the mitogen-activated protein kinase (MAPK) family. They also showed that the activation of ERK5 pathway in turn induced the phosphorylation of cell survival-transcription factors including CREB, ATF-1, and c-FOS in HK-2 human renal proximal tubular cells. Further study is necessary to see that the ERK5 pathway was also involved in the phosphorylation of CREB in our study by administering BIX02189, MAPK/ERK kinase inhibitor. The -150/-20 bp region of the StAR gene has been shown to harbor three SF-1-binding cis elements, namely SF-1/1, SF-1/2, and SF-1/3. Notably, the SF-1/3 region located between -106 and -92 bp upstream of the transcriptional start site has previously been shown to be more closely involved with both basal and cAMP-induced StAR gene expression (Montmny et al., 1986) and StAR promoter activity in comparison with the SF-1/1 and SF-1/2 sites (Manna et al., 2003).

To confirm whether CRE and SF-1/3 elements are important for the regulation of StAR expression by cadmium, EMSA was performed. The results of EMSA showed that DNA-protein complex formation with the CRE2 site of the StAR promoter (-96/-67) was increased upon cadmium exposure (Fig. 4B). However, cadmium decreased the binding of nuclear proteins to the SF-1/3 site (Fig. 4C). These results corresponded exactly to those obtained with immunoblotting. Treatment of K28 cells with cadmium resulted in an increase in the level of phosphorylated CREB and a decrease in the level of SF-1 (Fig. 3). Finally, the result shown in Fig. 5 provides strong evidence that cadmium-induced transcriptional activation of the StAR gene is mediated by phosphorylated CREB.

Cadmium is environmental contaminant, and mammals (including humans) can be easily exposed to cadmium in their surroundings. Accordingly, many researchers have studied cadmium, and a large body of data is available. Although the effect of cadmium on reproductive endocrinology has been reported, the molecular mechanism by which it affects steroidogenesis has remained unclear, especially in testicular Leydig cells. In this study, we have demonstrated that exposure of mouse testicular Leydig cells to cadmium results in an increase in StAR gene transcription. Moreover, we have presented evidence that cadmium up-regulates StAR gene transcription through phosphorylated CREB rather than SF-1. This study is the first report describing a molecular mechanism for cadmium-induced StAR gene regulation in testicular Leydig cells.
Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES


