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Effect of cadmium on cortisol production and 11β-hydroxysteroid dehydrogenase 2 expression by cultured human choriocarcinoma cells (JEG-3)

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ABSTRACT

Cadmium is a toxicant with known carcinogenic and endocrine disruptor effects. We have previously reported that prenatal exposure to cadmium may induce low birth weight which is associated to increased foetal exposure to glucocorticoids; both signals constitute "hallmarks" of developmental programming. Since the effect of cadmium on the glucocorticoid system of placental carcinogenic cells is unknown, in the present work, we studied the effect of acute low dose of cadmium on cortisol production and 11β-HSD2 expression and activity by cultured human choriocarcinoma cells (JEG-3). In addition, it was also evaluated whether those effects were related to the methylation index of the *HSD11B2* gene, which can be regulated by epigenetic mechanisms. Cells were incubated with low cadmium dose (0.5 and 1 μM) for 24 h and viability (MTT), cortisol production (EIA), 11β-HSD2 expression (qRT-PCR) and activity (radioassay), and methylation index of the *HSD11B2* gene were determined.

Results show lower cortisol concentrations in the incubation media of exposed cells, which were associated to increased 11β-HSD2 expression and activity and to a lower methylation index of the gene. These results suggest that cadmium-induced endocrine disruptor effects on JEG-3 cells could be mediated by changes in the methylation status of some target genes.

1. Introduction

Cadmium (Cd^{2+}) is widely dispersed in the environment mainly as result of pollution from a variety of sources, including mining, smelting, fossil fuel combustion, batteries, paints, and plastics, although tobacco smoke may be one of the most common sources of Cd^{2+} exposure affecting the general population (Bhattacharyya et al., 2000; Henson and Chedrese, 2004). Cadmium has been classified as a human carcinogen by the International Agency for Research on Cancer (Waisberg et al., 2003). It has been also recently identified as a new class of endocrine disruptor, being exposure to this metal linked to a wide range of detrimental effects on mammalian reproduction (Henson and Chedrese, 2004).

Previous studies from our laboratory have demonstrated that neonates delivered from mothers who smoked during pregnancy had reduced birth weight (BW), compared to those neonates from non-smoking mothers, a fact highly correlated with placental levels of Cd^{2+} (Ronco et al., 2005). Additionally, placentas of mothers delivering low birth weight (LBW) newborns showed significantly higher Cd^{2+} concentrations than placentas associated to normal birth weight neonates (Llanos and Ronco, 2009), suggesting that placental accumulation of heavy metals could be related to altered foetal growth mechanisms.

Many studies in humans and animals have shown that LBW is associated with altered hypothalamus–pituitary–adrenal (HPA) axis activity in later life (Kajantie et al., 2007). Thus, an embryo exposed to increased glucocorticoids (GC) concentrations shows LBW and deleterious metabolic consequences during the adult life (Mc Ternan et al., 2001; Seckl and Holmes, 2007). The adverse effects of GC exposure are partially related to changes in the expression of the glucocorticoid receptor (GR). The intracellular availability of glucocorticoids for binding to their receptors is modulated by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2; encoded by the *HSD11B2* gene), one of the most important regulators of GC in placental tissues. This enzyme, due to its localization to the syncytiotrophoblast layer of the placenta (Krozowski et al., 1995; Pepe et al., 1999), the site of maternal–foetal exchange, forms a functional barrier, restricting the free transfer of cortisol (human) or corticosterone (rat) between the maternal and foetal compartments by converting maternal cortisol to its inactive metabolite cortisone (human) and corticosterone to 11-dehydrocorticosterone (rat). Thus, the placental 11β-HSD2 protects the foetus from exposure to high levels of maternal glucocorticoid, being its enzymatic activity positively correlated with birth weight (Stewart et al., 1995; Murphy et al., 2002). Foetal growth restriction and low weight at birth may be involved in foetal/neonatal programming, which has

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been demonstrated to be mediated through epigenetic changes, being DNA methylation the most studied mechanism (Ozanne and Constancia, 2007; Szyf, 2009). Epigenetic alterations mediate some toxic effects of environmental chemicals like Cd^{2+} (Baccarelli and Bollati, 2009), with paradoxical effects on DNA methylation during Cd^{2+}-induced cellular transformation (Takiguchi et al., 2003).

Recently, Yang et al. (2006) reported that Cd^{2+} suppressed *HSD11B2* gene transcription, leading to reduced 11β-HSD2 expression and activity in normal cultured human trophoblast cells, suggesting that this could be one of the mechanisms involved in the Cd^{2+}-induced reduction in birth weight of smoker's newborns. Because 11β-HSD2 expression can be regulated by epigenetic mechanisms (Alikhani-Koopaei et al., 2004), we evaluated whether Cd^{2+} effects on 11β-HSD2 expression in cultured JEG-3 cells may be associated with changes in DNA methylation of its gene.

Accordingly, in this work, we studied the effect of low dose of cadmium on cortisol production and 11β-HSD2 expression and activity by cultured human choriocarcinoma cells, JEG-3, and determined the methylation index of the *HSD11B2* promoter gene.

2. Materials and methods

2.1. JEG-3 cultures

The American Type Culture Collection (ATTC, Manassas, VA) was the source of JEG-3 (HTB-36). The growth medium was Eagle's minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1.0 mM sodium pyruvate and a solution of 1% gentamycin–penicillin. The cells were routinely grown in 75 cm^2 vented flasks at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every other day. Cells were trypsinyzed with a solution of trypsin/EDTA (2.5 mg/ml/0.053 mM) in PBS pH 7.4, washed twice with PBS pH 7.4, centrifuged (at 1800*g* for 10 min) and finally seeded. To elaborate growth curves, percentage of adhesion and cell viability cells in exponential growth curves were used. Adherence was determined by the trypan blue method. The cells were passaged as required.

2.2. Assessment of cell viability

To evaluate the effects of Cd^{2+} on cell viability, the MTT reduction assay, which detects the active mitochondrial function, was used. The MTT assay depends upon the intracellular reduction of a soluble yellow tetrazolium dye (3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphenyl] tetrazolium bromide) to an insoluble blue–purple product (Mosmann, 1983). Only living cells with active mitochondria are able to generate a strong signal whereby MTT (pale yellow substrate) is converted into MTT formazan (dark blue insoluble crystals). For this assay, JEG-3 cells were incubated in the presence of different Cd^{2+} concentrations for 24 h at 34 °C in a humidified atmosphere of air:CO_2 (95:5%). After this time, 50 μl of an aqueous solution of MTT (5 mg/ml) was added to each well and the plate incubated for additional 4 h at 37 °C. Then, the cell suspensions were centrifuged at 450 *g* for 25 min. The supernatant (25 μl) was carefully aspirated avoiding disturbance of crystals already formed. DMSO (50 μl) was then added to dissolve the crystals and let for 5 min with shaking. Absorbance was measured at 540 nm in an ELISA reader. Appropriate controls were also run (i.e. medium and MTT, but not cells) (Ronco et al., 2001).

2.3. Assay of 11β-HSD2 activity—radiometric conversion assay

To study the effects of Cd^{2+} on 11β-HSD2 activity, cells were placed onto 12-well Corning plates and cultured to confluence. The cells were then cultured in serum-free medium for 24 h before treatment, and all the treatments were carried out under serum-free conditions. Cells in triplicate wells were then exposed to given doses of Cd^{2+} for 24 h; appropriate controls were also incubated for 24 h but in the absence of Cd^{2+}. At the end of treatments, cells were washed three times in serum-free medium. Then, the level of 11β-HSD2 activity in intact cells was determined by measuring the rate of cortisone conversion from cortisol, as described previously (Tremblay et al., 1999; Ronco et al., 2009). Briefly, the cells were incubated for 4 h at 37 °C in serum-free medium containing 100,000 cpm [^{3}H]-cortisol and 10 nM unlabeled cortisol. At the end of incubation, the medium was collected, and steroids were extracted with ethyl acetate. The extracts were dried, and the steroid residue was dissolved in 20 μl methanol containing 40 μg each of unlabelled cortisol and cortisone. The samples were quantitatively transferred to silica thin-layer plates and developed in chloroform–methanol (9:1, v/v). Bands containing labelled cortisol and cortisone were identified by UV light, then cut out into scintillation vials, and counted in a fluid scintillation counter (Packard 1600TR, Canberra Company). The rate of cortisol to cortisone conversion was calculated, and the blank values (defined as the amount of conversion in the absence of cells) were subtracted and expressed as a percentage of control. Results are shown as mean ± standard deviation (SD). Under conditions of the present study, the basal level of 11β-HSD2 activity in JEG-3 cells was approximately 10%.

2.4. Analysis of 11β-HSD2 mRNA

11β-HSD2 mRNA from control and Cd^{2+}-treated JEG-3 cells were quantified by real time PCR. Total RNA was extracted using TRI reagent kit (Ambion Austin, TX) according to the manufacturer's instructions. Total RNA was determined by spectrophotometry, measuring the absorbance ratio at 260/280 nm to evaluate the integrity, and the absorbance at 260 nm to determine its concentration. Single strand cDNA (ss-cDNA) was synthesized in a standard reverse transcription reaction using 1 μg of total RNA previously treated with DNAse, 200U M-MLV reverse transcriptase (Promega Madison, WI), and 0.5 μg oligo$(dT)_{15}$ (Promega). This ss-cDNA was used as template for quantitative PCR (qPCR) assays using water instead cDNA as negative control. Specific primers for 11β-HSD2 were: fw: 5′-GGCCAAGGTTTCCCAGTGA-3′ and rev: 5′-CAGGGTGTTTGGGCTCATGA-3′. Actin was used as housekeeping gene (Accession no. 42475962); fw: 5′-CCGTAAAGACCTCTATGCCA-3′; rev 5′-AAGAAAGGGTGTAAAACGCA-3′ having a product size of 352 pb. Gene transcript levels of all genes were quantified separately using the LightCycler® FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland) and a program with an activation step at 94 °C for 10 min followed by an amplification step with 45 cycles of 5 s at 94 °C, 5 s at 60 °C and 4 s at 72 °C. The mRNA in each RNA sample was quantified by the relative standard curve method and the relative amount of the specific genes was obtained and normalized to the values of β-*actin*. Data represent the averaged of two experimental replicates from six independent biological samples (Ronco et al., 2009).

2.5. DNA extraction and bisulphite treatment

DNA from control or Cd^{2+}-treated JEG-3 cells was extracted by the Wizard® Genomic DNA Purification Kit (Madison, WI, USA) and then treated with bisulphite. Briefly, in the bisulfite reaction, all unmethylated cytosines are deaminated and sulphonated to be converted into uracil bases, while 5-methylcytosine remains unaltered. Thus, the sequence of the treated DNA will differ depending on whether the DNA is originally methylated or unmethylated and, consequently, the primer sets were designed to spe-

cifically amplify either a bisulfite-sensitive or a bisulfite-resistant sequence.

The *HSD11B2* gene promoter region contains two CpG islands (GenBank accession no. U27317) which we considered in designing the specific sets of primers to detect CpG sites as either methylated or unmethylated. The DNA bisulfite treatment was essentially carried out as previously described by Clark and Frommer (1997) with modifications introduced by Warnecke and Clark (1999). DNA samples were treated with sodium metabisulfite for 16 h. The bisulfite reaction was desalted using a DNA clean-up column (Promega), as instructed by the manufacturer, and then bisulfite-treated DNA was eluted in 30 μl of dd H_2O. To verify that all genomic DNA was modified by bisulfite treatment fully methylated DNA was used as a control for bisulphite treatment (Epigendx, MA, USA). DNA was immediately used for PCR reaction or stored at −80 °C until additional use.

2.6. Methylation of the HSD11B2 gene promoter

Bisulfite-treated DNA from JEG-3 cells was amplified by PCR as previously described (Herman et al., 1996) using two sets of forward and reverse primers, which were designed to specifically detect CpG sites as being either methylated or unmethylated as previously described (Friso et al., 2008). Each PCR reaction for bisulfite-treated DNA was carried out in a total volume of 20 μl containing 10 μl of 1 × Promega master mix, 7 μl of DNAse-free H_2O, 1 μl of forward and reverse primers, and 1 μl of the template (Hirsch et al., 2008). PCR was performed by using hot start Taq polymerase (Invitrogen). For the forward primer 5′-GCGTAGCGAGATCGGGTGA-3′; for the reverse methylated primer 5′-ACTCTCCGCCGCGCC-3′; for the unmethylated forward primer 5′-TGTAGTGAGATTGGGTGAGTATTGGTT-3′ and for the unmethylated reverse primer 5′-TCAACTCTCCACCACACCCC-3′ were used. To verify the efficiency of the newly designed primers, both unmethylated and methylated primer sets were tested with bisulfite-treated fully unmethylated and fully methylated DNA which were also run during each PCR reaction together with the samples to be tested. Amplification products were resolved by gel electrophoresis and stained with ethidium bromide. The index of methylation was obtained by calculating the density (intensity/mm^2) of each band specific for methyl- and unmethyl-primers under UV light by a Gel Doc XR 170-8170 (Bio-Rad, Laboratories, Hercules, CA, USA). The index of methylation obtained by calculating the band density (intensity/mm^2) was expressed as the percent density of [methylated-band/(methylated-band + unmethylated-band)].

2.7. Statistical analysis

Data are expressed as means ± SD of at least four independent experiments ($n = 4$) run in triplicate. Results were evaluated by Student-*t* test for paired samples and for comparison among groups; parametric ANOVA followed by post hoc Bonferroni tests were applied.

3. Results

To determine whether Cd^{2+} exposure dose affect cell viability of JEG-3, we used the MTT assay (Fig. 1). Results, expressed as percentage of absorbance, indicated that low dose of Cd^{2+} (0.5 μM) did not change cell viability compared to control untreated cells. With 1 μM Cd^{2+}, a slightly, although no significantly increased absorbance was observed. These results indicate that under our incubation conditions, 1 μM Cd^{2+} do not affect JEG-3 cell viability after 24 h treatment. However, 1 μM Cd^{2+} decreased cortisol production by almost 60% (Fig. 2). To evaluate whether the reduced

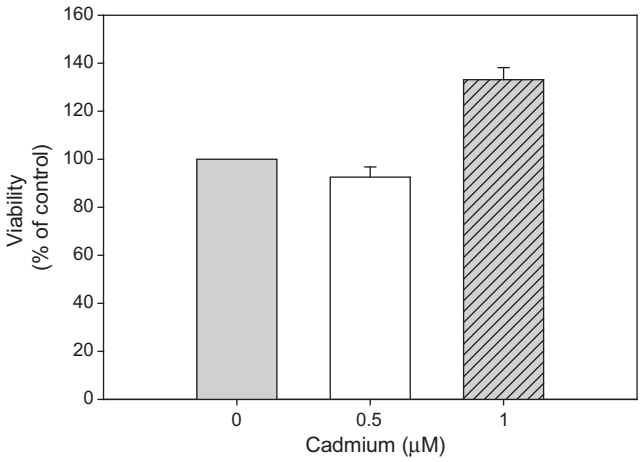

Fig. 1. Viability of JEG-3 cells incubated for 24 h with different Cd^{2+} concentrations. Results are the mean ± SD of the absorbance expressed as the percentage related to control of 4 different triplicate experiments related to the MTT reduction assay as described in Section 2.

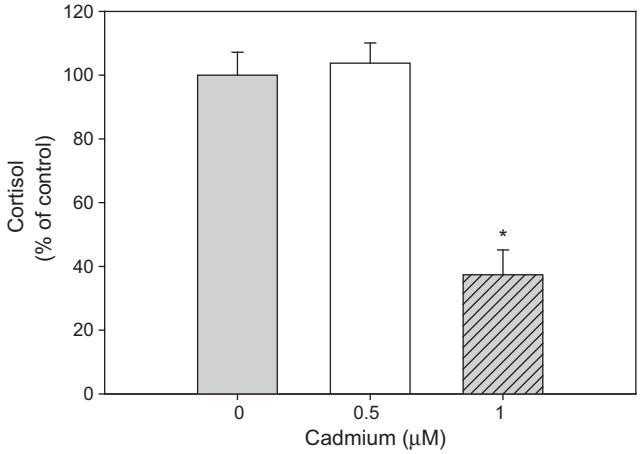

Fig. 2. Concentration of cortisol in the incubation media of JEG-3 cells after 24 h with different Cd^{2+} concentrations. Results are expressed as means ± SD of the percentage related to control of four different triplicate experiments, $^{*}p < 0.05$, ANOVA.

cortisol production was a consequence of a Cd^{2+} effect on the 11β-HSD2 activity, we measured the conversion of cortisol to corticosterone with a radioassay method. We found that 1 μM Cd^{2+} significantly increased 11β-HSD2 activity (Fig. 3), a result according to a reduction of cortisol and increased levels of corticosterone found in the incubation media. The increased 11β-HSD2 activity was correlated to an increased expression of the mRNA which was expressed as a percentage related to the expression of the *18S* rRNA (Fig. 4). Since the 11β-HSD2 expression may be related with the DNA methylation of the gene, we determined whether the increased 11β-HSD2 expression was related to the methylation status of its promoter sequence. As observed in Fig. 5, 1 μM Cd^{2+} induced a reduced methylation index of about 50%, concordant with a decreased expression of the gene.

4. Discussion

In this work we demonstrated that in cultured JEG-3 cells, a model for the human placental trophoblast, exposure to Cd^{2+} reduced the cortisol production and increased the 11β-HSD2 expression and activity; these findings were correlated with a reduced

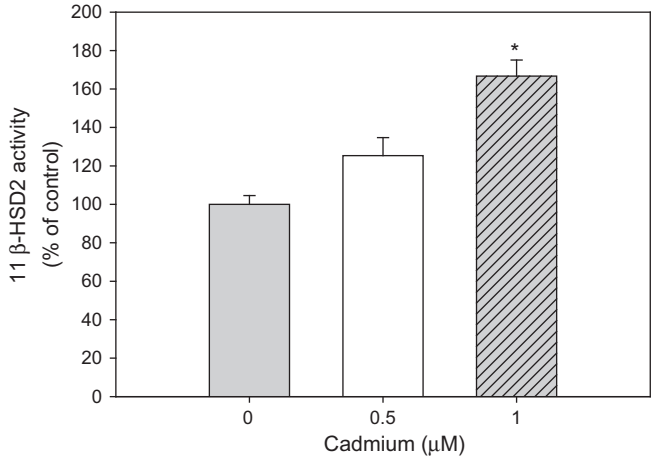

Fig. 3. Enzymatic activity of 11β-HSD2 in JEG-3 cells incubated for 24 h with different Cd^{2+} concentrations. Results are expressed as means ± SD of the percentage related to control of four different triplicate experiments, $^{*}p < 0.05$, ANOVA.

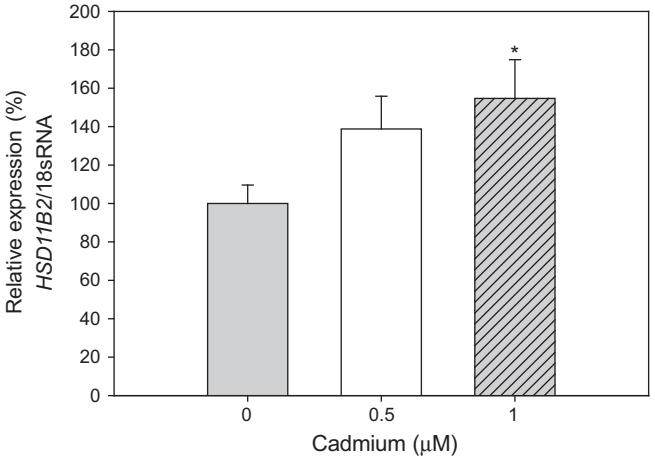

Fig. 4. Expression of 11β-HSD2 (mRNA) related to *18S* rRNA by real time PCR in JEG-3 cells incubated for 24 h with different Cd^{2+} concentrations. Results are expressed as means ± SD of the percentage related to control of four different triplicate experiments, $^{*}p < 0.05$, ANOVA.

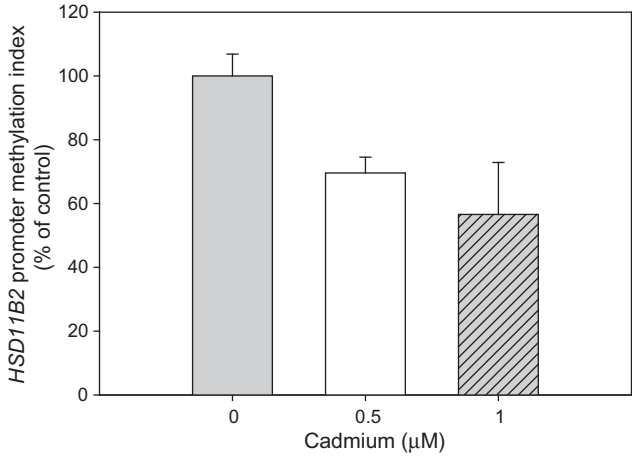

Fig. 5. Methylation of *HSD11B2* promoter of JEG-3 cells incubated with different concentrations of Cd^{2+} for 24 h. Results are expressed as methylation index and represent the percent density (intensity/mm^2) of [methylated-band/(methylated-band + unmethylated-band)]. Results are the mean ± SD of four different experiments run in triplicate, $^{*}p < 0.05$, ANOVA.

methylation index at the promoter sequence of the *HSD11B2* gene, suggesting that epigenetic mechanisms could be involved in the observed effects. Given that human placental trophoblast and JEG-3 cells endogenously produce similar factors (prostaglandins, leukotrienes) hormones and enzymes, included 11β-HSD2, the use of JEG-3 cells may represent an appropriate and relevant model to study human placental 11β-HSD2 action mechanism (Hardy et al., 1999).

The placental 11β-HSD2 enzyme, encoded by the *HSD11B2* gene, has emerged as a key player in controlling foetal development due to its localization to the syncytiotrophoblast layer of the human placenta (Krozowski et al., 1995; Pepe et al., 1999), serving as a functional barrier to protect the foetus from exposure to high levels of maternal glucocorticoids (Yang, 1997; Burton and Waddell, 1999) and to its involvement in birth weight (Stewart et al., 1995; Murphy et al., 2002).

Since mutations in the *HSD11B2* have been associated to foetal growth restriction and Cd^{2+} is also related to birth weight reduction, 11β-HSD2 has been considered a target for Cd^{2+}-induced weight reduction. In primary cultured normal human placental trophoblast cells, Yang et al. (2006) reported that Cd^{2+} suppresses *HSD11B2* gene transcription leading to a reduced 11β-HSD2 expression and activity. A reduced 11β-HSD2 expression has been associated to LBW neonates since placental cortisol concentrations cannot be inactivated appropriately and the final consequence for the foetus is an increased exposure to GC which affects birth weight. However, the endocrine disruptor effects of Cd^{2+} related to the GC system on placental cells already transformed are unknown.

The *HSD11B2* promoter comprises a highly G + C-rich (or GC-rich) core, contains more than 80% GC, lacks a TATA-like element, and has two typical CpG islands. This raises the possibility that CpG dinucleotide methylation may play a role in the cell type-specific and possibly in the epigenetically determined inter-individual variable expression of *HSD11B2*. Alikhani-Koopaei et al. (2004) reported that CpG methylation regulates the *HSD11B2* expression in vitro and in vivo, being hypermethylation of normally unmethylated CpG islands correlated with transcriptional repression.

With these antecedents, we studied the acute effect of low dose of Cd^{2+} on cortisol production and 11β-HSD2 expression and activity by cultured human choriocarcinoma cells (JEG-3) and evaluated whether those effects were related with the methylation index of the *HSD11B2* gene.

Unexpectedly, after 24 h of incubation with 1 μM Cd^{2+}, we observed a decreased production of cortisol in the media which was concordant with an increased 11β-HSD2 expression and activity and a reduced methylation index of the *HSD11B2* gene, indicating that *HSD11B2* expression and activity are inversely correlated with higher methylation status at the gene promoter. A strong negative correlation between the methylation status and gene expression of the *HSD11B2* promoter in JEG-3 cells was previously demonstrated (Alikhani-Koopaei et al., 2004). One mechanism by which promoter methylation affects expression of the corresponding gene is to modulate the binding of transcription factors (Tate and Bird, 1993). Recently, Baserga et al. (2010) demonstrated that intrauterine growth restriction affects renal 11β-HSD2 epigenetic determinants of chromatin structure, altering binding of a key transcriptional factor to the 11β-HSD2 promoter which was associated with down regulation of its mRNA expression.

Defects in methylation, including hypomethylation and hypermethylation, of genomic DNA or particular DNA sequences have been shown to be associated with the carcinogenic process, possibly as a factor in causation that facilitates aberrant under or over-

expression of oncogenesis linked genes (Zhao et al., 1997; Goodman and Watson, 2002). Understanding what modulates changes in the DNA methylation patterns during malignant transformation is an important issue in chemical carcinogenesis, including carcinogenesis by inorganics. For instance, DNA methylation changes appear to occur with various carcinogenic inorganic compounds, including arsenic (Zhao et al., 1997), nickel (Lee et al., 1998), and cadmium, with effects in DNA methylation and DNA methyltransferase (DNMT) activity (Takiguchi et al., 2003). Epidemiological studies have provided evidence that occupational and/or environmental exposure to Cd^{2+} is associated with human pulmonary cancers and possibly cancers in other tissues (Waalkes, 2000).

Although in this study, we did not compare the methylation status of the *HSD11B2* promoter gene of JEG-3 cells with normal trophoblast cells, we can infer that DNA methylation pattern between both type of cells, normal and tumour, may be different. In general, cells or tumours exhibit global hypomethylation during carcinogenic transformation when compared with their normal counterparts (Feinberg and Vogelstein, 1983; Esteller and Herman, 2002). A previous study demonstrated that in a normally non-tumourigenic liver cell line, Cd^{2+} was an effective inhibitor of DNMT initially leading to DNA hypomethylation (after 1 week exposure) and that, prolonged exposure (after 10 weeks) induced cell transformation including hyperproliferation, and increased invasiveness, and also DNA hypermethylation by enhancing DNMT activities (Takiguchi et al., 2003). In addition, in human embryo lung fibroblast (HLF) cells exposed to long-term low dose Cd^{2+}, both, the level of DNA methylation and DNMT activity were increased, suggesting that disruption of DNA methylation may be one of the possible underlying carcinogenic mechanisms of Cd^{2+} (Jiang et al., 2008).

In summary, this study demonstrates that an acute exposure (24 h) of a low dose of Cd^{2+} (1 μM) to cultured JEG-3 cells, induces decreased cortisol production associated with an increased 11β-HSD2 expression and activity. These effects of Cd^{2+} on choriocarcinoma cells are opposed to those observed in normal cultured trophoblast cells (Yang et al., 2006). In addition, this work suggests that an altered DNA methylation mechanism of the *HSD11B2* gene may be involved in these Cd^{2+}-induced endocrine disruptor effects. Additional studies are necessary to unambiguously demonstrate methylation changes in specific CpG islands of the *HSD11B2* promoter, as recently reported in renal tissue of growth restricted rats (Baserga et al., 2010). Cadmium exposure may affect the methylation pattern and consequent gene expression, depending of the type of cells: normal or tumour, the exposed Cd^{2+} dose, and the duration of the exposure.

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