Inhibition of CYP450scc expression in dioxin-exposed rat Leydig cells

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Abstract

Polychlorinated dibenzo-\textit{p}-dioxins, such as 2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin (TCDD) have been recognized as highly potent developmental and reproductive toxins. We have previously demonstrated effects of TCDD in modulating the expression of rat Sertoli cell secretory products and markers for cell–cell interaction. In this study, we examined the direct biological effects of TCDD in rat Leydig cell primary cultures. Mature rat Leydig cells were purified by Percoll gradient centrifugation and the cell purity was determined by 3\(\beta\)-hydroxysteroid dehydrogenase (3\(\beta\)-HSD) staining and a testosterone induction assay. To examine TCDD-induced biological consequences, we measured the changes in the secretion of progesterone and testosterone, as well as transcript levels of some selected steroidogenic enzymes (i.e. StAR, P450scc, 3\(\beta\)-HSD and CYP17\(\alpha\)), in TCDD/human chorionic gonadotropin (hCG) co-treated cells. Our results indicated that TCDD (0·2 or 2 ng/ml) treatment significantly suppressed hCG (5 or 10 ng/ml)-induced testosterone secretion. The suppressive effect aligned with a reduction of progesterone secretion (\(P<0·05\), as well as a decrease of P450scc mRNA and protein expression (\(P<0·05\)). The mechanistic action of TCDD was found to be via the reduction of cellular cAMP levels in the hCG-treated cells. This observation was further confirmed, as the TCDD-mediated suppressive effect could be reversed by dibutyryl cAMP co-treatment. The data indicate that TCDD can modulate cAMP signaling in rat Leydig cells to affect the process of steroidogenesis.

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Introduction

2,3,7,8-Tetrachlorodibenzo-\textit{p}-dioxin (TCDD), the most toxic man-made compound, is mostly generated as an unintentional by-product from many industrial processes, such as waste incineration, pesticide manufacturing, pulp and paper bleaching. Since dioxin is a fat-soluble compound with a semi-volatile nature, it is ubiquitously present in the environment and can be bioaccumulated in food-chains. In general, the mechanistic toxicity of TCDD is mediated by the nuclear translocation of an aryl hydrocarbon receptor (AhR)/AhR nuclear translocator complex that binds to dioxin-responsive elements or by interfering with various signaling molecules in cells (Reyes et al. 1992, Matsushita et al. 1993, Safe 1995, Schmidt & Bradfield 1996). On the basis of its action, TCDD is recognized as an endocrine disruptor which can have adverse impacts on mammalian reproductive and developmental processes. Its detrimental effects on gonadal function in humans and animals are well documented.

Using \textit{in utero} and lactational exposure studies in rodent models, toxicities of TCDD (\(\mu\)g/kg) at the early stage of animal development were reported. In female progenies, TCDD disrupted regular estrous cycles and inhibited the onset of ovulation (Li et al. 1995, Salisbury & Marcinkiewicz 2002). In male offspring, reduction of sperm count \textit{per cauda} epididymis as well as increases in the number of abnormal sperm produced in adulthood were observed (Mably et al. 1992, Bjerke & Peterson 1994, Faqi et al. 1998). Most of the male progeny were characterized by a reduced size of sex accessory glands (Mably et al. 1992, Bjerke & Peterson 1994, Gray et al. 1995, Theobald & Peterson 1997). Accompanying this, other studies scrutinized the biological consequences for postnatal animals that received an i.p. injection of TCDD (\(\mu\)g/kg body weight). Those studies demonstrated that TCDD altered the process of testicular steroidogenesis and caused a reduction of Leydig cell volume and number (Johnson et al. 1992, 1994, Wilker et al. 1995). Detrimental effects on Sertoli and germ cells of rat testes, such as reduction of the intercellular contact of neighboring cells, disruption of germ cell development, decrease of spermato genesis, depletion of antioxidant enzymes and increase in the levels of lipid peroxidation were observed (Rune et al. 1991\textit{a}, Chahoud et al. 1992, Mably et al. 1992, Peterson et al. 1993, Latchoumycandane et al. 2002\textit{a,b}). It is generally believed that the adverse effects exerted by TCDD on male reproductive functions are manifold and
pleiotropic. Yet the understanding of reproductive toxicities of TCDD for testicular function has mostly relied on the in vivo approach of using animal models. Complications in pharmacokinetic distribution, as well as secondary effects attributed to other unidentified factors, may make it difficult to decipher the direct mechanistic toxicities of TCDD to the cells. This is particularly true as AhR is widely expressed in various tissues of animals (Dohr et al. 1996, Thomae et al. 2004). Therefore it is necessary to adopt cell models to determine direct biological effects of TCDD to validate the in vivo findings.

With the benefit of hindsight, the present study aimed to reveal the mechanistic role of TCDD on reproductive disorders with special reference to mammalian Leydig cells. It is well known that Leydig cells play a crucial role in synthesizing testosterone and regulating the process of spermatogenesis. Alteration of cell function can lead to adverse effects on testicular functions. In this study, we aimed to elucidate the effect of TCDD on the process of steroidogenesis and testosterone secretion in Leydig cells. To decipher the mechanistic activities, TCDD-stimulated cytochrome P4501A1 (CYP1A1) expression and mRNAs of the key steroidogenic enzymes, as well as the level of progesterone and testosterone secretion, were measured.

Materials and Methods

Primary culture of rat Leydig cells

Leydig cells were isolated from testes of 90- to 120-day-old sexually mature male Sprague–Dawley rats and were cultured for 2 days as described elsewhere (Biegel et al. 1993). All experimental animals were housed, handled and used in accordance with the University guidelines. Briefly, testes were collected and decapsulated from five male rats and were then digested in M199 medium containing 0.5 mg/ml collagenase and 1% BSA in Erlenmeyer flasks in a 34 °C oscillating incubator at 100 r.p.m. for 15 min. Digested cell suspensions were transferred to a 50 ml tube and kept on ice for 2 min to allow tubules to settle. The supernatant containing Leydig cells was filtered through a3 40·5 mg/ml collagenase and 1% BSA in Erlenmyer flasks and was then digested in M199 medium containing 1% BSA in Erlenmeyer flasks in a 34 °C oscillating incubator at 100 r.p.m. for 15 min. Digested cell suspensions were transferred to a 50 ml tube and kept on ice for 2 min to allow tubules to settle. The supernatant containing Leydig cells was filtered through cell strainers (70 μm nylon; Falcon, BD Biosciences, Franklin Lakes, NJ, USA) and the cells were then centrifuged at 350 g for 20 mins at 4 °C. The pellet was re-suspended in 10 ml M199 and loaded on top of a step Percoll gradient (5, 30, 58 and 70%) (Sigma). The cell was then centrifuged at 800 g for 30 mins at 4 °C. Three layers of cell were apparent; the enriched fraction of Leydig cells was found in the third layer. The cells were collected, washed with M199 twice and were finally re-suspended in phenol red-free-DMEM/F12 (1:1) medium supplemented with 10% charcoal stripped fetal bovine serum and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) were exposed for 24 h to one of the following treatments: (i) 1–50 ng/ml hCG (Sigma); (ii) 0·2–2000 pg/ml TCDD (Cambridge Isotope Laboratories Inc., Andover, MA, USA); (iii) hCG (1–50 ng/ml) + TCDD (0·2–2000 pg/ml); (iv) 2 mM dibutyryl cAMP (dbcAMP) (Calbiochem) + TCDD (200 or 2000 pg/ml) + hCG (5 or 10 ng/ml); (v) hCG (1–10 ng/ml) + 0·1 μM dexamethasone (DEX) (Calbiochem, San Diego, CA, USA); and (vi) dimethylsulfoxide (DMSO) (Sigma) solvent control. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction method. The viability of the control and treated cells was over 90%.

The treated cells were used for the measurement of mRNAs for steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage enzyme (P450scC), 3β-HSD-1, cytochrome P450–17α enzyme (CYP17α), CYP1A1 and glyceraldehyde–3-phosphate dehydrogenase (GAPDH). Conditioned media were assayed for progesterone and testosterone.

Progesterone determination

The conditioned media were assayed for progesterone content using a progesterone ELISA kit.
(ICN Pharmaceuticals) according to the manufacturer’s instructions. Briefly 25 µl sample/standard, 100 µl working progesterone–HRP conjugate reagent and 50 µl rabbit anti-progesterone reagent were mixed sequentially in the wells and incubated at 22 °C for 90 min. The wells were rinsed five times with distilled water and mixed with 100 µl TMB solution followed by 20 min incubation at 22 °C. The reaction was then stopped and the absorbance was read at 450 nm within 15 min.

**Effects of TCDD on mRNA levels of steroidogenic enzymes and CYP1A1**

StAR, P450scc, 3β-HSD-1, CYP17α, CYP1A1 and GAPDH PCR products were generated by PCR of total RNA derived from the isolated Leydig cells. The primers were designed on the basis of the published sequence of StAR (GCAGGAGCAACTGAGTG-forward and TG ATGGTCTCAGGACGACC-reverse) (Ronen-Fuhrmann et al. 1998); P450scc (CCTCT GTGAATACTGGTG ATAGGG-forward and AGCTGGGCAACATGGGAG TCA-reverse); 3β-HSD-1 (ACTGGGAAATTTCTCCA TAGCC-forward and TTCTTCCAGCTGAAC ATA GTGG-reverse) (Dasmahapatra et al. 2000); CYP17α (CAGGAAGCCAAAGTCACGGTG-forward and TGT TGAACGAGTATACAGACATGA-reverse); CYP1A1 (CCTCTTGGGAGCTGGTTTG-forward and TGC GTGCGGATGGTTGAAG-reverse); and GAPDH (ATGGGAAGCGTGTTGCAC-forward and TCC ACCACCTTGTGTTGT A-reverse). The PCR fragments for StAR (246 bp), P450scc (200 bp), 3β-HSD-1 (405 bp), CYP17α (150 bp), CYP1A1 (230 bp), and GAPDH (200 bp) were purified, subcloned into pCRII-TOPO (Invitrogen) and subjected to dideoxy sequencing for verification. The purified plasmids were quantified and the respective copy numbers were calculated.

**Real-time PCR**

The treated cells were dissolved in TRIZOL Reagent (GIBCO/BRL) and total RNA was extracted according to the manufacturer’s instructions. Purified RNA with a 1·6–1·8 A260/A280 ratio was used in this study. Real-time PCR was conducted for mRNA quantification. Briefly, 1 µg total cellular RNA was reverse transcribed using an iScript cDNA synthesis kit (BioRad). Quantified standards (10^4–10^8) and sample CDNA were analyzed by an iCycler iQ real-time PCR detection system using iQ SYBR Green Supermix (Bio-Rad). The copy number for each sample was calculated and the data were normalized using the expression level of GAPDH mRNA. The PCR conditions were 95 °C for 3 min and 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. Fluorescent signals were captured at 82 °C, the occurrence of primer dimers and secondary products was inspected using melting curve analysis. Control amplifications were done either without RT or without RNA. Following PCR amplification, the reaction products were run at 100 V on a 1% agarose gel with 0·5 µg/ml ethidium bromide to determine product specificity. All glass- and plastic-ware were treated with diethyl pyrocarbonate and autoclaved.

**Western blot analysis**

The treated cells were washed with two or three changes of cold PBS. Adherent cells were scraped from the plastic surface and transferred to a microcentrifuge tube. The cells were pelleted and resuspended in 30–50 µl cold lysis buffer containing 250 mM Tris–HCl, pH 8·0, 1% NP-40 and 150 mM NaCl. After 10 min incubation on ice, the lysed cells were pelleted and supernatants were assayed for protein concentration (DC Protein Assay Kit II; Bio-Rad Pacific Ltd). Samples were subjected to electrophoresis in NuPage 4–12% Bis–Tris gradient gels (Invitrogen). Gels were blotted onto a PVDF membrane. Western blotting was conducted using rabbit antibodies to P450scc (Chemicon Int., Temecula, CA, USA), followed by incubation with HRP-conjugated goat anti-rabbit antibody and was developed with chemiluminescent reagent (Western-lightening Plus; PerkinElmer Life Sciences, Boston, MA, USA). The blot was then washed in PBS and re-probed with rabbit anti-actin antibody (Sigma).

**cAMP measurement**

Cells were exposed for 1·5 h to one of the following treatments: (i) 5 ng/ml hCG; (ii) hCG+TCDD (200 or 2000 pg/ml); or (iii) DMSO solvent control. Cell lysates prepared in 0·1 M HCl were used for cAMP measurement according to the manufacturer’s instructions with a cAMP immunoassay kit (R&D Systems, Minneapolis, MN, USA).

**Statistical analysis**

Drugs treatments were performed in triplicate in the same experiments and individual experiments were repeated at least three times. All data are represented as means ± S.E. Statistical significance was tested by Student’s t-test. Groups were considered significantly different if P<0·05.

**Results**

**Characterization of the isolated Leydig cell model**

The four-step Percoll gradient centrifugation yielded cells of three major populations. The top layer (layer-1, p<1·035 g/ml) consisted of small round cells and cell debris. Layer-2 (p>1·076 g/ml) was composed of spermatozoa and a small fraction of Leydig cells. The cells in
the third layer (ρ~1·085 g/ml) were over 90% Leydig cells, as they were positively stained by the 3β-HSD method (Fig. 1A and B). The basal testosterone secretion level of the layer-3 cells was about 55-fold higher than that of layer-1 and -2 cells (Fig. 1C). The layer-3 cells were also highly sensitive to hCG stimulation, in which over a 25-fold induction of testosterone secretion was detected. Layer-1 and -2 cells, however, showed no or moderate responses to the stimulation. According to the 3β-HSD staining and testosterone induction assay, the layer-3 cells were confirmed as Leydig cells and were used for the latter part of the study.

Effect of TCDD on testosterone secretion of hCG-treated Leydig cells

Treatment of the Leydig cell culture with different doses of hCG (1–50 ng/ml) produced a dose–response induction curve of testosterone secretion (Fig. 2A). Significant reduction of testosterone secretion was detected in cells co-treated with 5 or 10 ng/ml hCG and 200 or 2000 pg/ml TCDD (Fig. 2B). However, there were no significant change in the ED₅₀ and Vₘₐₓ response of the cells to hCG stimulation. The profile of inhibition was different from the action of DEX, whereas the ED₅₀ were
considerably reduced (Fig. 3). Intriguingly, the TCDD-mediated reduction of testosterone secretion can be counteracted by a higher dose of hCG (50 ng/ml) co-treatment. The countereffect can also be imitated by 2 mM dbcAMP (data not shown). To elucidate the underlying mechanism of the TCDD-elicited reduction of testosterone secretion, we measured the mRNA expression levels of some key steroidogenic enzymes, including StAR, P450scc, 3β-HSD-1 and CYP17α using the real-time PCR method (Fig. 4A). In the hCG-treated cells, expression of the steroidogenic enzymes was significantly stimulated. In the co-treatment with 200 or 2000 pg/ml TCDD and 5 or 10 ng/ml hCG, TCDD significantly reduced the expression of P450scc (−70%). A decrease in protein level was consistently demonstrated

**Figure 2** Testosterone induction curves upon hCG or hCG+TCDD treatment in rat Leydig cell cultures. The cells were incubated for 24 h in 10% charcoal dextran stripped serum/DMEM/F12 (1:1) containing different doses of hCG (1–50 ng/ml). A dose–response induction curve of testosterone secretion is shown in (A). When the hCG-stimulated cells were co-treated with an increasing dose of TCDD (0–2000 pg/ml), no change in the ED50 and Vmax responses to hCG stimulation were observed. However, significant reductions of testosterone secretion were measured at the conditions of 5 or 10 ng/ml hCG and 200 or 2000 pg/ml TCDD co-treatments (B). Data (means ± s.e.m.) are from three separate experiments.

**Figure 3** Effect of DEX on testosterone secretion in hCG-induced rat Leydig cells. The cells were incubated for 24 h in 10% charcoal dextran stripped serum/DMEM/F12 (1:1) containing DEX (0.1 μM). Significant reductions of testosterone secretion and an increase in ED50 response to hCG stimulation were found. Data (means ± s.e.m.) are from three separate experiments.

**Figure 3** Effect of DEX on testosterone secretion in hCG-induced rat Leydig cells. The cells were incubated for 24 h in 10% charcoal dextran stripped serum/DMEM/F12 (1:1) containing DEX (0.1 μM). Significant reductions of testosterone secretion and an increase in ED50 response to hCG stimulation were found. Data (means ± s.e.m.) are from three separate experiments.
by Western blotting (Fig. 4B). Since P450scc mediated the synthesis of progesterone, we assayed the progesterone levels in the conditioned media of the TCDD/hCG co-treated cells. Consistently there was a significant reduction of progesterone levels in the cells (Fig. 4C).

It is well known that Leydig cell steroidogenesis can be stimulated by luteinizing hormone (LH), whereas cAMP plays an important role in the regulation (Pon et al. 1986, Pon & Orme-Johnson 1986, Epstein & Orme-Johnson 1991, Clark et al. 1995, Luo et al. 1998). To determine if TCDD treatment can modulate cellular cAMP levels, we measured its levels in hCG and hCG/TCDD co-treated cells. Elevated cellular cAMP levels were measured at 1 h after hCG treatment. The maximal elevated level was detected at 1·5–2 h after treatment (Fig 5A). Treatment of the hCG-induced cells with 200 and 2000 pg/ml TCDD significantly reduced the cellular cAMP level ($P<0.05$) (Fig. 5B). A significant reduction of P450scc protein level was detectable at 8 h after TCDD/hCG treatment (Fig. 5C).

**Figure 4** Effect of hCG and hCG+TCDD on (A) the mRNA expressions of selected steroidogenic enzymes (i.e. STAR, P450scc, 3β-HSD-1 and CYP17α), (B) P450scc protein levels and (C) progesterone levels of the rat Leydig cell culture. The cells were incubated for 24 h in 10% charcoal dextran stripped serum/DMEM/F12 (1:1) containing 5 ng/ml hCG and hCG+0·2 or 2 ng/ml TCDD. (A) For real-time PCR, total RNA of each sample was reverse-transcribed and analyzed by an iCycler iQ real-time PCR detection system using iQ SYBR Green Supermix. P450scc mRNA levels in hCG+TCDD-treated cells were significantly (*$P<0.05$) reduced. Note that the x-axis is on a log 10 scale. (B, C) Significant reduction of P450scc and progesterone levels were detected after TCDD+hCG co-treatment (*$P<0.05$). Data (means ± S.E.M.) are from three separate experiments.

**Discussion**

Our previous studies have demonstrated effects of TCDD in modulating the expression of Sertoli cell secretory products (i.e. Mullerian inhibiting factor, 17β-estradiol) and markers (i.e. sertolin and testin) for cell–cell interaction (Lai et al. 2005). The studies suggested that TCDD exposure can interfere with intercellular communication and possibly disrupt the process of spermatogenesis. These findings prompted the present investigation to examine possible effects of TCDD on Leydig cell functions, particularly for the synthesis and secretion of testosterone. In the first part of this study, the modulating effects of TCDD on hCG-induced testosterone secretion were examined. Treatment of the cells with hCG produced a dose–response curve of testosterone induction. In the co-treatment studies using doses of 0·2 or 2 ng/ml TCDD and 5 or 10 ng/ml hCG, a significant reduction of progesterone (43%) and testosterone (32%) levels was observed. The TCDD-mediated reduction of progesterone and testosterone production, however, can be completely abolished at a higher dose (50 ng/ml) of hCG treatment. This observation is in agreement with other reports using animal models, whereas high doses of hCG could alleviate TCDD-mediated inhibition of Leydig cell functions (Wilker et al. 1995, Mandal et al. 2001) and prevented the TCDD-elicited reduction of cytochrome P450 enzymes (Ruangwises et al. 1991). To delineate the underlying mechanism of TCDD-mediated inhibition of testosterone synthesis, we measured the changes in the expression of some key steroidogenic enzymes. Our results demonstrated that there was a 70% reduction of P450scc mRNA levels in the TCDD/hCG co-treated cells. Similar findings were observed in mice that were i.p. administrated with 100 ng/g TCDD (Fukuzawa et al. 2004). The TCDD-mediated inhibition of steroidogenic
enzymatic activities seems not to be gender specific, as a P450scc mRNA reduction in TCDD (3·1 nM)-exposed rat primary granulosa cells was reported (Dasmahapatra et al. 2000).

In this study, we observed that the effective dose of TCDD to modulate Leydig cell functions falls in the ng/ml level. The molar concentrations for 0·2 and 2 ng/ml used in this study are equivalent to 0·62 and 6·2 nM respectively. The concentrations of TCDD used are comparable or even lower than other reports that had demonstrated the inhibitory effect of TCDD on Leydig cell function or testosterone production in animal models (Rune et al. 1991b, Chahoud et al. 1992, Johnson et al. 1992, 1994, Mably et al. 1992, Peterson et al. 1993, Wilker et al. 1995, Latchoumycandane et al. 2002a,b). In addition, it is comparable to others’ cell-culture systems where nanomolar levels of TCDD exposure were needed to cause noticeable effects on signaling molecules (Shibazaki et al. 2004, Vogel et al. 2004).

In the present study, we have demonstrated that TCDD-mediated inhibition can be completely impeded by higher dose of hCG co-treatment. Consistently, the hCG countereffect can be imitated by cAMP treatment. These results shed light onto the possible mechanistic action of TCDD in interfering with the process of steroidogenesis. With the benefit of hindsight, we measured and compared the change of cellular cAMP levels in hCG co-treated Leydig cells. Intriguingly we can detect an acute cellular effect of TCDD at 2 h after treatment in hCG co-treated cells, whereas the cellular cAMP level was significantly reduced. Accompanying this early event, a significant reduction of P450scc protein levels was measured.

Hitherto, our data demonstrated that the significant reduction of the secreted progesterone and testosterone levels in the Leydig cells were attributed to the reduced expression of P450scc. It is a rate-limiting enzyme mediating the conversion of cholesterol to pregnenolone.
(Stocco 2001). It is likely that the reduced enzyme level was due to the decrease of cellular cAMP at the early phase of hCG stimulation. In a mouse fibroblast model, Vogel et al. (2004) had demonstrated that the effect of TCDD on cellular cAMP level was AhR-dependent, whereas the binding of the immunophilin-like protein (XAP2) to cAMP-specific phosphodiesterase (PDE4A5) modulated cAMP signaling. Other studies had also proposed that the effect of TCDD on steroidalogenic enzymes was AhR-dependent (Sugawara et al. 2001, Fukuzawa et al. 2004). Taken together, we suggest that the TCDD-mediated reduction of cellular cAMP levels in this study is possibly AhR-dependent. The potential roles of LH receptor, adenylate cyclase or cAMP-specific phosphodiesterase in the mechanistic action of TCDD have not been addressed in this study.

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