Direct and indirect impact of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on adult mouse Leydig cells: an in vitro study.

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Direct and indirect impact of 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) on adult mouse Leydig cells: an in vitro study.

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Short title: Impact of TCDD on cultured Leydig cells

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Abstract:

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related substances are ubiquitous environmental pollutants that exert adverse effects on reproductive processes. In testis, Leydig cells which produce testosterone are under hormonal and local control exerted by cytokines including TNFα. Using mouse Leydig primary cell cultures as a model, we studied the effects of TCDD on the steroidogenic outcome of Leydig cells and the gene expression levels of Ccl5 and Cxcl4, previously shown to be target genes of TCDD in testis. We found that TCDD did not alter the steroidogenic outcome of Leydig cells but that it up-regulated Cxcl4 gene expression levels. TCDD also impacted Ccl5 gene expression when cells had been co-treated with TNFα. TCDD action probably initiated with binding to the aryl hydrocarbon receptor (AhR) present on Leydig cells. TCDD regulated the gene expression levels of AhR (transient down-regulation) and its repressor AhRR and Cyp1b1 (up-regulation). The trophic human chorionic gonadotropin (hCG) hormone did not impact AhR, its repressor AhRR or Cyp1b1 but it opposed the TCDD-enhanced AhRR mRNA levels. Conversely, TNFα stimulated AhR gene expression levels. Collectively, it is suggested that the impact of TCDD on expression of target genes in Leydig cells may operate under the complex network of hormones and cytokines.

Key words: Dioxin; Leydig cell; in vitro; AhR; chemokine

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), as well as halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) and related compounds, are highly persistent environmental pollutants. Dioxins are by-products from incineration processes and pesticide production, and thus they are not intentionally produced. They are considered endocrine disruptors (Diamanti-Kandarakis *et al.* 2009; Hotchkiss *et al.* 2008; Lundqvist *et al.* 2006), i.e. chemical substances that interfere with the endocrine systems of humans and wildlife. As such, they are of high concern for human health because endocrine disrupters are suspected to be responsible for apparent changes seen over recent decades, including congenital malformations, cancer and declining sperm counts (Skakkebaek *et al.* 2001; Toppari *et al.* 1996). Dioxins are not genotoxic but cause a broad spectrum of adverse effects including hepatotoxicity, immune system suppression, developmental toxicity, and skin defects. TCDD mediates its toxicity by binding to the aryl hydrocarbon receptor (AhR) and subsequent alteration of the expression of target genes which exhibit dioxin response elements in their promoter moiety including the cytochrome CYP1A1 (Barouki *et al.* 2007; Mimura and Fujii-Kuriyama 2003). Apart from phase I and phase II enzymes of the detoxification machinery, microarray studies allowed identifying chemokines as potential new target genes in liver (Boutros *et al.* 2009; Boutros *et al.* 2008) but also in testis (Rebourcet *et al.* 2010), suggesting that TCDD exposure may induce inflammatory responses in addition to detoxifying processes. Evidences also accumulated showing that inflammatory cytokines including the tumor necrosis factor α (TNFα) were downstream targets of AhR signalling pathways (Haarmann-Stemmann *et al.* 2009).

In the adult testis, Leydig cells, which are fully differentiated cells, produce the male primary steroid hormone, testosterone, in response to their trophic Luteinizing Hormone, LH. In addition to being under hormonal control, Leydig cells are under a local control exerted by autocrine and/or paracrine factors such as cytokines and chemokines (Benahmed 1997; Guazzone *et al.* 2009). Leydig cells are presumed to be somewhat resistant to various types of chemotoxicants due to the insufficiency of CYP1A1 expression and activity as demonstrated elsewhere (Chung *et al.* 2007). The xenobiotic metabolizing enzyme CYP1B1 can also be induced by treatment with AhR agonists (Shimada and Fujii-Kuriyama 2004) and in contrast to CYP1A1, CYP1B1 is a predominantly extra-hepatic CYP enzyme. For example, it is highly expressed in adrenals and gonads (Leung *et al.* 2009). However, its regulation by PAHs in steroidogenic tissues is controversial (Deb *et al.* 2010; Mandal *et al.* 2001).

In the present study, we were interested in delving further into the adverse effects caused by TCDD on testis physiology focusing on Leydig cells using a model of primary cultures. We demonstrated that Leydig cells expressed AhR, its repressor AhRR and Cyp1B1 genes, and that they are under TCDD regulation. The chemokines Cxcl4 and Ccl5, previously identified *in vivo* as target genes for TCDD (Rebourcet *et al.* 2010) were directly (Cxcl4) or indirectly (Ccl5) regulated by TCDD. However, TCDD did not alter steroidogenic outcome of Leydig cells. Collectively, our data support the hypothesis that Leydig cells and thus the testis is not inert with respect to toxic insult. It also suggested that TCDD impacted the expression of target genes in Leydig cells under the complex network of hormones and cytokines.

2. Materials and Methods

2.1. Leydig cell preparation

Swiss male CD-1 mice aged of 8 weeks were purchased from Harlan Laboratories France (Gannat, France). Animals were killed by CO_2 asphyxia. Testes were removed and immediately used for cell preparations. All procedures were performed with the approval of the Regional Committee of Ethics for Animal Experiments.

Leydig cells were isolated and cultured in HAM's F12-DMEM (PAA Laboratories, Les Mureaux, France) at 32°C in an humidified atmosphere of 5% CO_2 as previously described (Carreau *et al.* 1988; Mazaud Guittot *et al.* 2008). Briefly, testes were decapsulated and digested with 0.25 mg/ml collagenase at 32°C for 10 min. The digestion procedure was stopped by dilution with fresh medium. Interstitial cells were collected in the supernatant and the pellet containing aggregates of Sertoli and germ cells was discarded. Interstitial cells were purified on a discontinuous Percoll density gradient (layers of 21, 26, 34, 40 and 60% Percoll). The gradient was centrifuged at 800 g for 30 min. The interface between 40 and 60% was collected and washed with medium to remove the Percoll. The presence of 3-β hydroxysteroid dehydrogenase (3beta-HSD) activity was revealed by a histochemical technique described in details elsewhere (Bilinska *et al.* 1997) using the anti-3beta-HSD antibody provided by Dr I. Mason (Reproductive and Developmental Sciences Division, Edinburgh, UK). It was used to determine the purity of Leydig cells (95%). Contamination by intestitial macrophages did not exceed 3%. Cells were resuspended in fresh culture medium supplemented with 2% Fetal Calf Serum (FCS). They were plated in 12-well plates (400,000 cells/well). After 24 h, culture media were replaced with serum-free culture media.

2. 2 Leydig cell treatment

Two days after plating, culture media were renewed and 0.5 ml of fresh serum-free culture media was added per well in the presence or not of different products listed below for the time periods indicated in the text. These products included human chorionic gonadotropin (hCG; 100 ng/ml, Organon Schering-Plough, France) to mimic the effect of LH, dibutyryl cyclic AMP (dbcAMP; 0.2 mM, Sigma-Aldrich, France), recombinant Tumor Necrosis Factor alpha (TNFα; 20 ng/ml; Peprotech France), Interleukin 1 alpha (IL1α; 20 ng/ml, Peprotech France), Lipopolysaccharides (LPS; 10 microg/ml; Sigma-Aldrich, France). Cytokines were used at a 20 ng/ml concentration to maximally stimulate Leydig cells (Benahmed 1997; Hong *et al.* 2004). For 2,3,7,8-tetrachlorodibenzo-p-dioxin (ref ED-901-C; LGC Promochem, Molscheim France), stock solutions were prepared in dimethylsulfoxide (DMSO) and appropriately diluted in culture medium. DMSO volume content per well was 0.04 microl in a final volume of 0.5ml per well. Cell viability was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] reduction method according to manufacturer's instructions (Promega, Charbonnières, France). Treatments did not alter cell viability which was over 95%. Doses of TCDD higher than 25 nM resulted in loss of cell viability and were not used in the present study.

2.3 Testosterone Measurement by Enzyme Immunoassay

Testosterone levels in the culture media were measured directly with a testosterone immunoassay kit purchased from Cayman Chemical Company (Interchim, Montlucon France). The sensitivity of the assay was 6 pg/ml.

2.4 RNA extraction, Reverse Transcription and Quantitative PCR (RT-qPCR)

Total RNA was extracted from cultured Leydig cells using TRI Reagent (Applied Bioystems, Courtaboeuf, France). RNA integrity was determined with the Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Massy, France). Briefly, First-strand cDNAs were synthesized from 1 µg of total RNA in the presence of 100 U of Superscript II (Invitrogen, Eragny, France) and a mixture of random hexamers and oligo(dT) primers (Promega). Real-time PCR assays were performed in duplicates for each sample using a Rotor-Gene™ 6000 (Corbett Research, Mortlake, Australia), as described (Rebourcet *et al.* 2010). The list of the primers used (Invitrogen, Eragny, France) is available on Table 1.

Briefly, PCR was performed in the presence of 0.4 µM of specific sense and antisense primers and 10 µl Absolute QPCR SYBR Green ROX mix (Thermo Fisher Scientific, Courtaboeuf, France), in a total volume of 20 µl. After the initial denaturation step of 15 min at 95°C, the reaction conditions were 40 cycles of 95°C for 15 sec, 55 to 62°C (depending on the primers, Table 1) for 10 sec, and 72°C for 20 sec. Melting curve analyses were performed immediately following the final PCR cycle to verify the specificity of the PCR product by checking its Tm. Rpl19 (ribosomal protein L19) gene was chosen for normalizing target genes in the testis. It was consistently and reproducibly expressed in all samples, and it did not vary following treatments including TCDD treatment. Relative quantification was made by the standard curve method for both target and housekeeping gene (endogenous control) in each sample. A series of dilutions of calibrator sample (external standard) was included in each experiment in order to generate an external standard curve. Then the concentration of the target in each sample was divided by the concentration of the housekeeping gene in each sample, to correct for sample heterogeneity and variability of detection.

2.5 Statistical analysis

All experiments have been performed at least three times, with independent preparations of cells. Data were expressed as means ± SEM and the comparisons between treatments were made by one-way analysis of variance (ANOVA) followed by the post hoc Fisher PLSD test for multiple comparisons. A *p* value of less than 0.05 was considered significant. All statistical analyses were done with the aid of Statview 5.0 software package (SAS Institute Inc. Cary, NC 27513).

3. Results

3.1 Steroidogenic outcome of Leydig cells treated with TCDD

Addition of TCDD (25 nM) to Leydig cells did not alter basal and dbcAMP-induced testosterone production after 6 h of treatment (Fig. 1a). RT-qPCR experiments were also developed to survey expression levels of genes that mediate the first steps in steroidogenesis including the Steroidogenic acute regulatory protein (StAR) and Cyp11a1 in Leydig cells treated with TCDD, and stimulated or not with hCG or dbcAMP for 6 h. Within these conditions, TCDD had no impact on StAR (Fig. 1b) or Cyp11a1 (data not shown) gene expression levels.

3.2 Gene expression of AhR, the repressor AhRR and Cyp1b1, and their regulation by TCDD and hCG or dbcAMP

TCDD mainly exerts its action through binding to AhR. This step is followed by a rapid activation of target genes including the repressor AhRR, Cyp1A1 and Cyp1B1 (Abel and Haarmann-Stemmann 2010; Barouki *et al.* 2007). Cyp1A1 is not expressed at all in the testis (Chung *et al.* 2007; Rebourcet *et al.* 2010) nor was it induced following TCDD challenging in Leydig cells (data not shown). We found that Leydig cells expressed AhR, the repressor AhRR and Cyp1b1. TCDD added for 6 h regulated significantly ($p<0.05$) the level of mRNAs encoding AhR (a 40% decrease), AhRR (a 7 fold-increase) and Cyp1b1 (a 2.5 fold-increase) (Fig. 2). Addition of hCG 100 ng/ml or dbcAMP 0.2 mM had no effect on AhR and Cyp1b1 gene expression levels (Fig. 2). However, hCG significantly halved the TCDD-induced increase in Ahrr gene expression levels after 6 h of treatment (Fig. 2b). This effect was also observed after 24 h of treatment with a 4-fold decrease in hCG and TCDD co-treated cells versus TCDD-treated cells (data not shown). Addition of dbcAMP also opposed TCDD enhancement of AhRR gene expression levels after 6 h of treatment, indicating the involvement of Protein Kinase A (Fig. 2b).

3.3 Expression of the chemokines Ccl5 and Cxcl4 in primary culture of Leydig cells and their regulation by TCDD

We previously demonstrated that Ccl5 and Cxcl4 were 2 chemokines whose expression levels were altered in rat testis exposed to TCDD at gestational age 15. In addition, CCL5 immuno-reactivity was mostly confined to Leydig cells (Rebourcet et al., 2010). We therefore determined by RT-qPCR that the chemokines Ccl5 and Cxcl4 were expressed at the mRNA level in the mouse cultured Leydig cells. In addition, we found that Cxcl4 (Fig. 3a) but not Ccl5 (Fig. 3b) gene expression levels were significantly up-regulated by TCDD and dbcAMP. Combined treatment resulted in additive effects (Fig. 3a).

3.4 Local regulation of the TCDD targeted genes by TNFα

We investigated potential interactions between TNFα and TCDD in the model of primary culture of Leydig cells. Cells were treated for 24 h. We found that TNFα stimulated significantly ($p<0.05$) AhR but not AhRR gene expression levels (Fig. 4). The negative effects of TCDD on AhR gene expression levels observed at 6 h of treatment (Fig. 2a) were

not observed at 24 h of treatment. However, TCDD-treated cells did no longer respond to TNFα by enhanced AhR mRNA levels (Fig. 4a). Gene expression levels of Cxcl4 and Ccl5 were also investigated. The addition of TNFα, IL1α and LPS, described as stimulators of chemokines (Graves and Jiang 1995; Nomiyama *et al.* 2010) up-regulated Cxcl4 (Fig. 5a) and Ccl5 (Fig. 5b) gene expression levels in primary culture of Leydig cells. TNFα was a potent stimulator for the two chemokines (4 and 4.4-fold increase for Cxcl4 and Ccl5 respectively, $p<0.05$) (Fig. 5). By contrast, IL1α stimulated more Cxcl4 (7-fold increase) than Ccl5 (1.8-fold-increase) gene expression. LPS stimulated more Ccl5 (8-fold increase) than Cxcl4 (2.8-fold increase) gene expression (Fig. 5). Extending data presented in Fig. 3, we observed that TCDD could significantly ($p<0.05$) oppose the TNFα-induced increase in Ccl5 mRNA levels (Fig. 5b); with no effect on TNFα-induced increase in Cxcl4 mRNA levels (Fig. 5a). A summary of all the observed effects is provided on Table 2.

4. Discussion

Understanding the detrimental effects caused by TCDD is limited using animal models, and primary cell culture models represent useful tools to decipher mechanistic toxicities. In the present study, we were interested in delving further into the adverse effects caused by TCDD on Leydig cell physiology focusing on AhR dependent genes and chemokines, recently identified as TCDD target genes.

We first demonstrated that TCDD did not impact the steroidogenic outcome of Leydig cells assessed through measurement of StAR and Cyp11a1 expression levels, and testosterone production in Leydig cells cultured in basal conditions and under stimulation by the trophic hormone or dbcAMP. These data are consistent with previous in vitro studies showing no direct effect of TCDD on steroidogenesis in MA-10 mouse Leydig tumor cells and adult rat Leydig cells (Mandal et al., 2001). These data are also consistent with our previous study indicating no change in testosterone levels in the testes of rats exposed in utero to low doses of TCDD (Rebourcet et al., 2010), whereas reduced testosterone levels were observed in adult rats treated with very high doses of TCDD (Johnson *et al.* 1994; Kleeman *et al.* 1990; Moore *et al.* 1985).

Leydig cells recovered from fully mature animals are known to express AhR (Schultz *et al.* 2003), but no reports have yet identified the TCDD regulation of AhR in Leydig cells nor determined if the repressor AhRR was present in Leydig cells and regulated by TCDD. We found that TCDD down-regulated transiently the expression of AhR gene, and

enhanced the expression of AhRR gene, suggesting negative feedback to limit AhR signalling effect. Indeed, it has been previously shown that AhRR functions as a naturally occurring dominant-negative factor limiting AhR signalling pathway (Abel and Haarmann-Stemmann 2010; Mimura *et al.* 1999). Hence, AHRR is an important determinant of tissue specific responsiveness to TCDD, and strong evidences have been reported on an inverse relationship between AHRR expression and sensitivity to induction of xenobiotic-metabolizing enzymes caused by TCDD (Korkalainen *et al.* 2004). The data presented herein i.e., high expression of AhRR and no detectable Cyp1a1 induction are in keeping with these observations. Interestingly, in conditions of increased gene expression levels of StAR following hCG or dbcAMP stimulation, genes encoding AhR and AhRR had unchanged expression levels. We next investigated the combined effects of TCDD with hCG/dbcAMP or TNFα. Indeed, TNFα may originate from the systemic circulation or from the neighbouring interstitial macrophages (Guazzone et al., 2009). We found that addition of hCG which protects testis function against TCDD in adult rats (Lai *et al.* 2005), while not acting directly on AhR or AhRR gene expression levels, attenuated the TCDD-induced stimulation of AhRR. Unlike hCG, TNFα did not reduce TCDD action on the AhRR gene expression levels, and enhanced AhR expression levels. Altogether, these data are in keeping with previous studies showing that gonadotrophins and TNFα exert inverse effects on Leydig cells (Benahmed 1997; Guazzone *et al.* 2009).

Testicular Cyp1B1 is localized predominantly in Leydig cells (Ge *et al.* 2005) and subject to developmental regulation in rat testis (Leung *et al.* 2009). Herein, we found that TCDD enhanced Cyp1b1 expression levels in Leydig cells but hCG and dbcAMP had no effect. These data do not support previous studies (Deb and Bandiera 2011; Deb *et al.* 2010). Such a discrepancy may result from the use of primary cultures of adult Leydig cells in our system versus Leydig cell lines in Deb and collaborators. In addition, adult Leydig cells are less responsive to gonadotropins with aging (Midzak *et al.* 2009) and, Deb and collaborators (Deb *et al.* 2010) also observed that *in vivo* treatment with TCDD increased testicular CYP1B1 protein levels in adult rats. The meaning of the enhancement of Cyp1b1 by TCDD is unclear. TCDD is not metabolized by CYP1B1. Besides, CYP1B1 is not required for mammalian development, reproductive vitality and physiological homeostasis as demonstrated by the absence of major phenotype in Cyp1b1-null mice (Buters *et al.* 1999). More studies are required to elucidate the role CYP1B1 may play in Leydig cell physiology.

It has been recently demonstrated that TCDD apart from the classical battery of genes of the detoxification machinery, may provoke inflammatory adverse effects (Haarmann-Stemmann *et al.* 2009). For example, it was demonstrated that TCDD could enhance TNFα secretion in a model of cultured adipocytes (Nishiumi *et al.* 2010) as well as in the serum of rats orally treated with TCDD (Ciftci *et al.* 2010). It was also shown that dioxin through binding to AhR could stimulate Ccl5 gene expression in an *in vitro* model of endometriosis (Yu *et al.* 2008; Zhao *et al.* 2002), Ccl1, a chemokine involved in cardiovascular diseases and inflammatory or allergic processes (N'Diaye *et al.* 2006) and Ccl2 in multiple organs of mice (Vogel *et al.* 2007). TCDD also inhibited Cxcl12 gene expression and its receptor Cxcr4 in MCF-7 breast cancer cells (Hsu *et al.* 2007). Chemokines are a subset of small chemotactic cytokines produced by both immuno-competent cells and non-immune cells. They are secondary pro-inflammatory mediators that are induced by cytokines such as interleukin-1 or TNFα (Graves and Jiang 1995; Nomiyama *et al.* 2010). We here found that Leydig cells could express Ccl5 and Cxcl4, and that these chemokines responded to stimulation by LPS, IL1α and TNFα. In addition, depending on the conditions (presence or not of TNFα), these two chemokines were targeted by TCDD in primary cultures of adult mouse Leydig cells, consistently with our previous in vivo findings (Rebourcet et al., 2010).

Collectively, we demonstrated that TCDD can directly and indirectly impact in vitro adult Leydig cell function through alteration in the gene expression levels of chemokines as well as AhR signalling pathway, supporting the hypothesis that Leydig cells and thus the testis are not inert with respect to toxic insult. If considering that TCDD may stimulate TNFα secretion, our data raise the hypothesis that the resulting AhR activation in testis following an *in vivo* TCDD insult might be the sum of a direct action by TCDD itself on Leydig cells and an indirect inflammatory response with elevated systemic TNFα subsequently activating AhR signalling pathway in Leydig cells. More insight into the molecular mechanisms behind the influence of TCDD on Leydig cells will require determining the involvement of the TNFα signalling transduction pathways, specifically the involvement of NF-κB and mitogen-activated protein kinases (MAPKs). Indeed, and depending on cell types and culture conditions, it was shown that hCG could attenuate NF-κB activation and cytokine expression (Huber *et al.* 2007), and trigger transient MAPK kinase activation (Brion *et al.* 2011). Therefore, integrated responses to TCDD would be highly dependent on the cell type, its local and hormonal environment.

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BLM and DN designed the study. DN, MAC and BLM performed the cultures and treatment. NV, MV, EL performed the QPCR dosages. DR performed the immunostaining and cell viability tests. AJ and MV performed the western blotting experiments. BLM, DN and MB analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Table 1: List and sequence of primers used for PCR analysis

Gene symbol	Accession no.	forward 5'-3'	Reverse 5'-3'	Size (bp)	Hybrid. T(°C)
RPL19	NM_009078.2	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC	195	58
CCL5	NM_031116.3	CTTGCAGTCGTCTTTGTCAC	GACTAGAGCAAGCAATGACAG	158	58
CXCL4 (PF4)	NM_019932.4	AGCGATGGAGATCTTAGCTGTGTG	GGTCCAGGCAAATTTTCCTCCCATTC	160	58
AHR	NM_013464.4	TCATCTGGTTTCCTGGCAATGAAT	ATAAGCTGCCCTTTGGCATC	245	62
AHRR	NM_009644.2	TCAGGGGACAAACAGATAGG	CTCAAGTGTACTGGTGTCTC	220	55
CYP1B1	NM_012940.1	GCAGCCGCCTTCCTGGTAGC	CCACGCGCCCTGTCCCTACT	116	60
STAR	NM_000349	ATGCAGAAGGCCTTGGGCAT	AACACCTTGCCCACATCTGG	113	60
CYP11A1	NM_019779	ACAAGCTGCCCTTCAAGAAC	TCCTTGATGCTGGCTTTGAG	223	58

The size of the expected PCR fragment in base pairs (bp) and the hybridation temperature (Topt) for annealing are reported.

Table 2: Summary of the effects described

	Stimuli added in vitro		
Genes surveyed	**TCDD**	**hCG/dbcAMP**	**TNFα**
Star and Cyp11a1	no effect	strong up-regulation	not determined
AhR	down-regulation at 6 h, not at 24 h; TCDD opposed TNFα-effect on AhR at 24 h	no effect	up-regulation at 24 h
AhRR	up-regulation at 6 & 24 h	no effect per se; can oppose TCDD-effect on AhRR at 6 & 24 h	no effect
Cyp1b1	up-regulation at 6 h	no effect	not determined
Cxcl4	up-regulation at 24 h	up-regulation at 24 h	up-regulation at 24 h
Ccl5	no effect per se; can oppose TNFα-effect at 24 h	no effect	up-regulation at 24 h

FIGURE LEGENDS:

Figure 1: Testosterone production (a) and relative gene expression of StAR (b) in primary cultures of Leydig cells recovered from 8-week old mouse testes treated for 6 h with various factors and assessed by EIA and RT-qPCR analysis, respectively. Factors added included TCDD 25 nM, dbcAMP (0.2 mM) and hCG 100 ng/ml. Testosterone production is expressed in ng/ml. StAR levels were normalized using Rpl19 and are expressed relatively to controls arbitrarily fixed to 1. Values are the mean ± SEM of n= 4 separate experiments. a, $p<0.05$ versus control. Boxes in black correspond to controls and boxes in grey indicate treatment with TCDD. Small window highlights testosterone production in basal conditions.

Figure 2: Relative expression of Ahr (a), Ahrr (b) and Cyp1b1 (c) in primary cultures of Leydig cells recovered from 8-week old mouse testes treated for 6 h with various factors and assessed by RT-qPCR analysis. Factors added included TCDD (25 nM), hCG (100 ng/ml), or dbcAMP (0.2 mM). Ahr (a), Ahrr (b) and Cyp1b1 (c) levels were normalized using Rpl19 and are expressed relatively to controls arbitrarily fixed to 1. Values are the mean ± SEM of n= 4 separate experiments. a, $p<0.05$ versus its respective control; b, $p<0.05$ versus TCDD-treated samples in (b). Boxes in black correspond to controls and boxes in grey indicate treatment with TCDD.

Figure 3: Relative expression of Cxcl4 (a) and Ccl5 (b) in primary cultures of Leydig cells recovered from 8-week old mouse testes treated for 24 h with various factors and assessed by RT-qPCR analysis. Factors added included TCDD (25 nM), dbcAMP (0.2 mM) or the two. Cxcl4 (a) and Ccl5 (b) levels were normalized using Rpl19 and are expressed relatively to controls arbitrarily fixed to 1. Values are the mean ± SEM of n= 6 separate experiments. a, $p<0.05$ versus its respective control; b, $p<0.05$ versus dbcAMP and TCDD-treated cells. Boxes in black correspond to controls and boxes in grey indicate treatment with TCDD.

Figure 4: Relative expression of Ahr (a) and Ahrr (b) in primary cultures of Leydig cells recovered from 8-week old mouse testes treated for 24 h with various factors and assessed by RT-qPCR analysis. Factors added included TCDD (25 nM), TNF α (20 ng/ml) or the two. Ahr and Ahrr levels were normalized using Rpl19 and are expressed relatively to controls arbitrarily fixed to 1. Values are the mean ± SEM of n= 8 separate experiments. a, $p<0.05$

versus its respective control; b, $p<0.05$ versus TCDD or TCDD and TNF α-treated cells in (b). Boxes in black correspond to controls and boxes in grey indicate treatment with TCDD.

Figure 5: Relative expression of Cxcl4 (a) and Ccl5 (b) in primary cultures of Leydig cells recovered from 8-week old mouse testes treated for 24 h with various factors and assessed by RT-qPCR analysis. Factors added included LPS (10 µg/ml), Il1α or TNF α (20 ng/ml), or TCDD (25 nM) or TNFα and TCDD in co-treatment. Cxcl4 (a) and Ccl5 (b) levels were normalized using Rpl19 and are expressed relatively to controls arbitrarily fixed to 1. Values are the mean ± SEM of n= 9 separate experiments. a, $p<0.05$ versus its respective control. B, $p<0.05$ versus TNFα –treated cells in (b). Boxes in black correspond to controls and boxes in grey indicate treatment with TCDD.

Figure 1

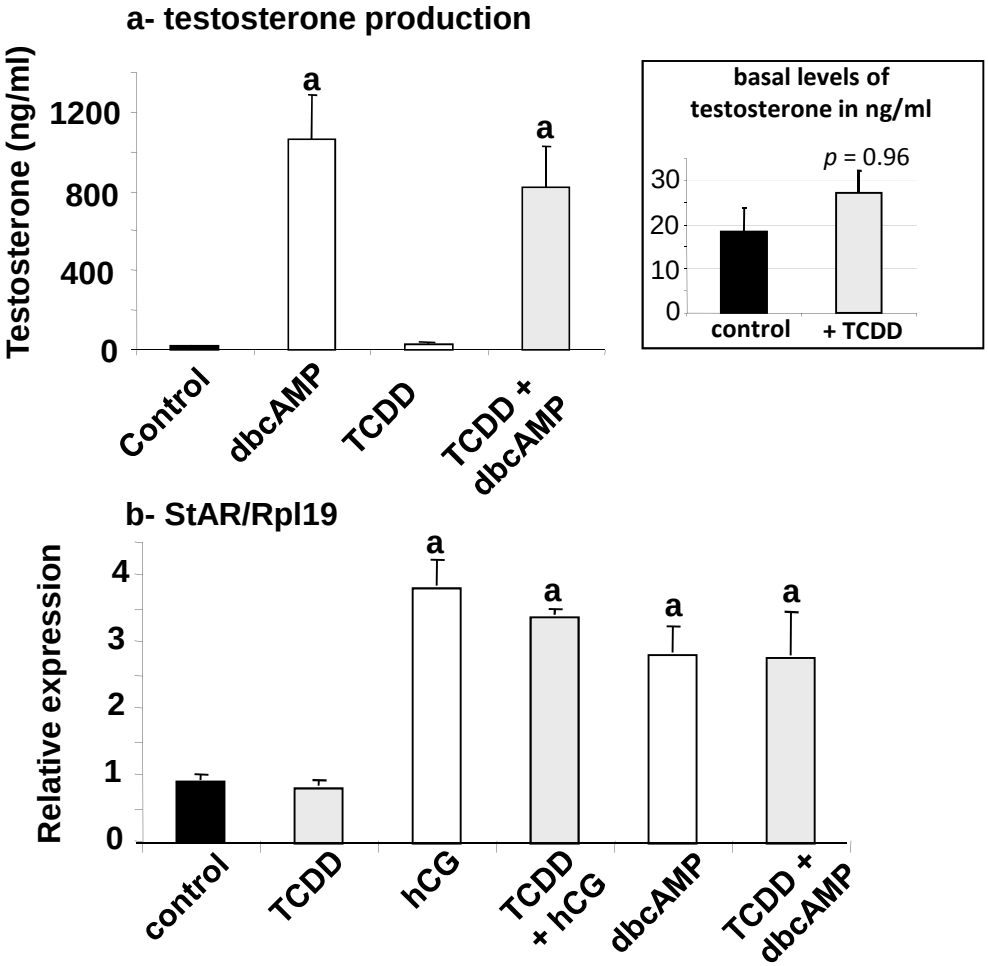

a- testosterone production
Testosterone (ng/ml)
1200
800
400
0
a
a
Control
dbcAMP
TCDD
TCDD + dbcAMP
basal levels of testosterone in ng/ml
p = 0.96
30
20
10
0
control
+ TCDD
b- StAR/Rpl19
Relative expression
4
3
2
1
0
a
a
a
a
control
TCDD
hCG
TCDD + hCG
dbcAMP
TCDD + dbcAMP

Figure 2

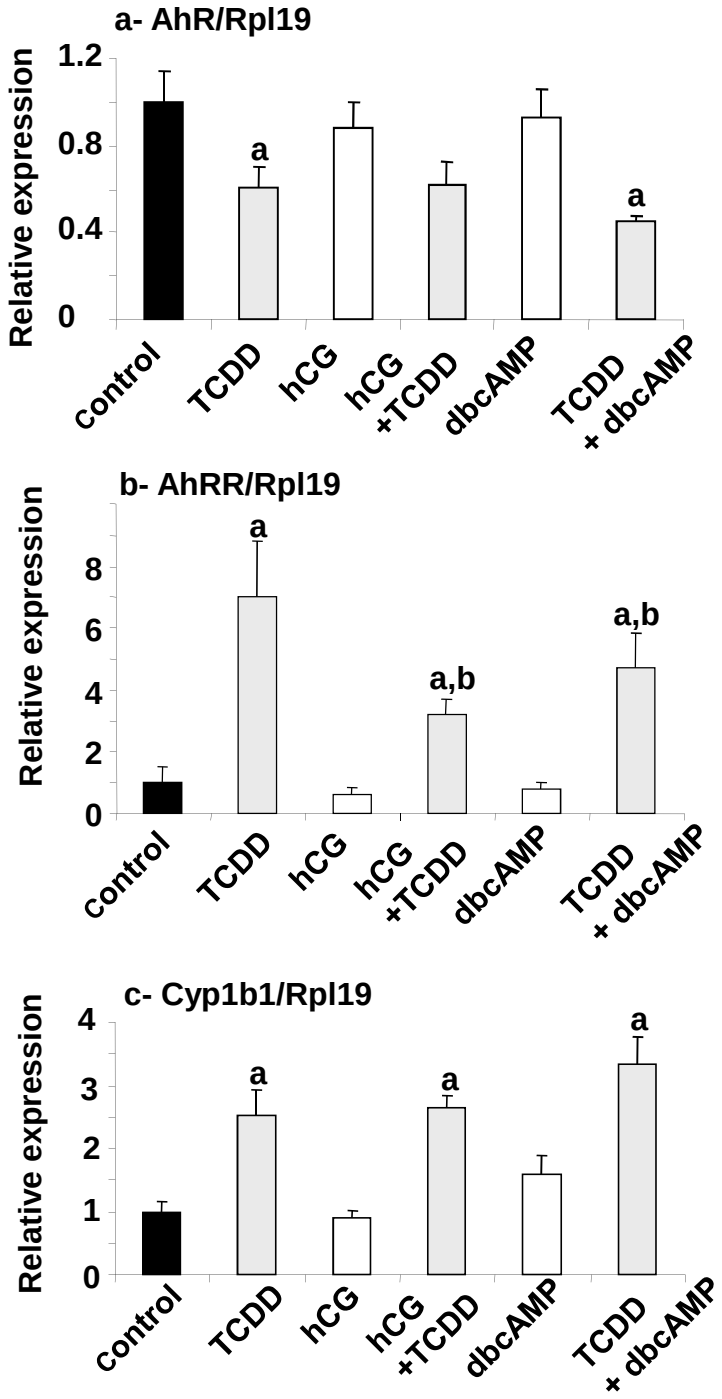

a- AhR/Rpl19
Relative expression
1.2
0.8
0.4
0
a
a
Control
TCDD
hCG
hCG +TCDD
dbcAMP
TCDD + dbcAMP
b- AhRR/Rpl19
Relative expression
8
6
4
2
0
a
a,b
a,b
Control
TCDD
hCG
hCG +TCDD
dbcAMP
TCDD + dbcAMP
c- Cyp1b1/Rpl19
Relative expression
4
3
2
1
0
a
a
a
Control
TCDD
hCG
hCG +TCDD
dbcAMP
TCDD + dbcAMP

Figure 3

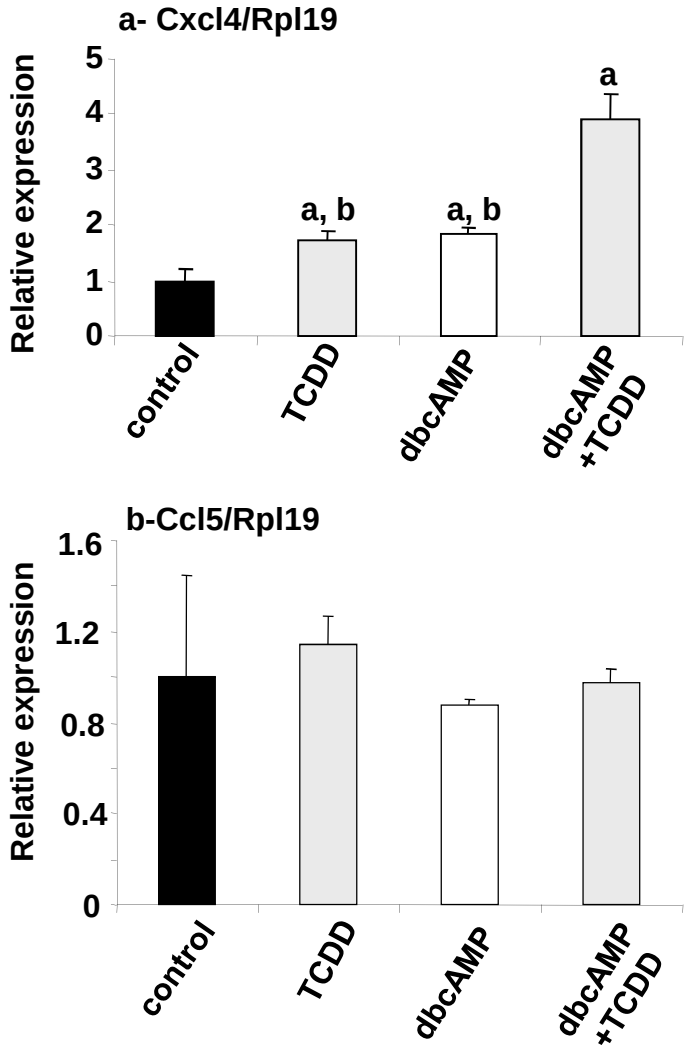

a- Cxcl4/Rpl19
Relative expression
5
4
3
2
1
0
a, b
a, b
a
control
TCDD
dbcAMP
dbcAMP +TCDD
b-Ccl5/Rpl19
Relative expression
1.6
1.2
0.8
0.4
0
control
TCDD
dbcAMP
dbcAMP +TCDD

Figure 4

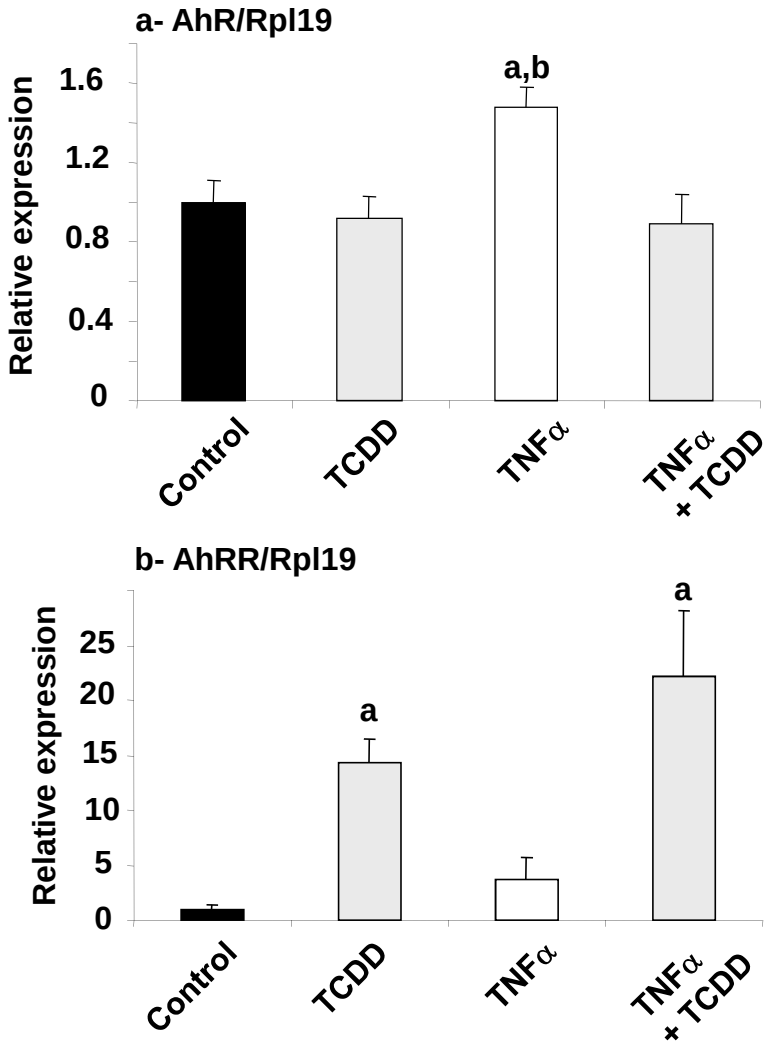
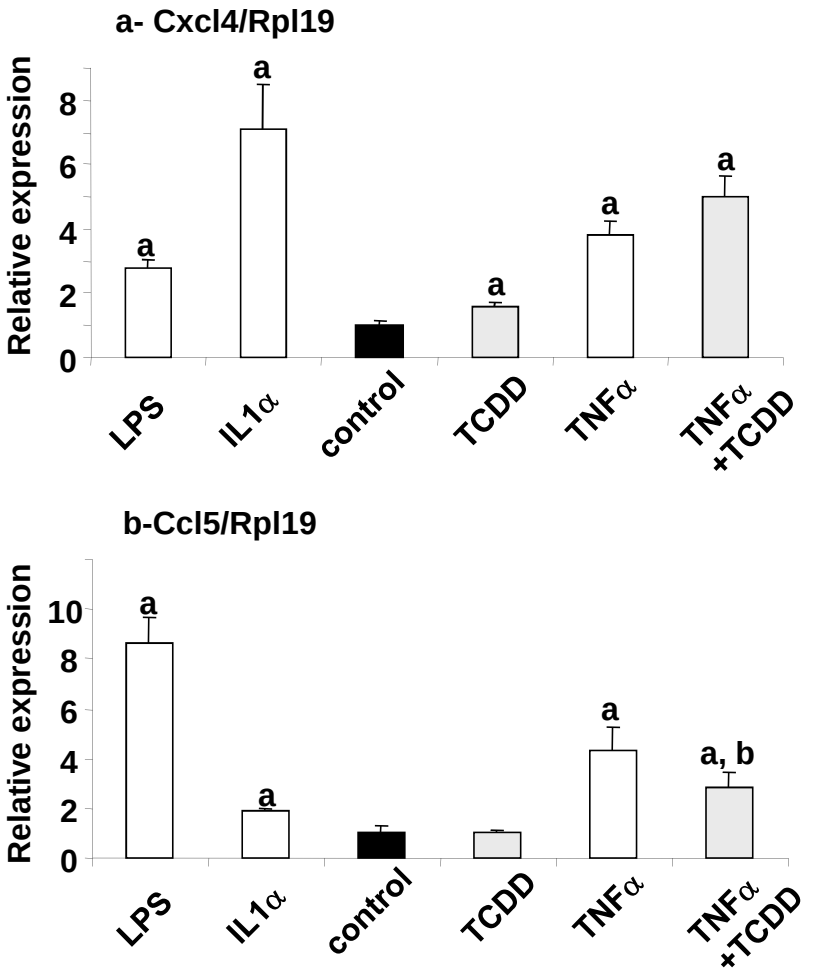

a- AhR/Rpl19
Relative expression
1.6
1.2
0.8
0.4
0
a,b
Control
TCDD
TNFα
TNFα + TCDD
b- AhRR/Rpl19
Relative expression
25
20
15
10
5
0
a
a
Control
TCDD
TNFα
TNFα + TCDD

Figure 5


a- Cxcl4/Rpl19
Relative expression
0
2
4
6
8
a
a
a
a
a
LPS
IL1α
control
TCDD
TNFα
TNFα +TCDD
b-Ccl5/Rpl19
Relative expression
0
2
4
6
8
10
a
a
a
a, b
LPS
IL1α
control
TCDD
TNFα
TNFα +TCDD