Toxicology Letters 207 (2011) 251–257

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

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

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

Danielle Naville[a,b,c], Diane Rebourcet[a,b,c,1], Marie-Agnès Chauvin[a,b,c], Nathalie Vega[a,b,c], Audrey Jalabert[a,b,c], Michèle Vigier[a,b,c], Emmanuelle Loizon[a,b,c], Martine Bégeot[a,b,c], Brigitte Le Magueresse-Battistoni[a,b,c,*]

[a] *INSERM, U1060, CarMeN, Laboratoire Lyonnais de Recherche en Cardiovasculaire, Métabolisme, Diabétologie et Nutrition, F-69921 Oullins, France*
[b] *Université Lyon-1, F-69000 Lyon, France*
[c] *INRA, UMR1235, F-69921 Oullins, France*

ARTICLE INFO

Article history:
Received 29 July 2011
Received in revised form
20 September 2011
Accepted 21 September 2011
Available online 29 September 2011

Keywords:
Dioxin
Leydig cell
In vitro
AhR
Chemokine

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.

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., 2008, 2009) 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

* Corresponding author at: INSERM U1060 – INRA U1235, Faculté de Médecine Lyon Sud, 165 Chemin du Grand Revoyet, 69921 Oullins, France. Tel.: +33 426 235 917; fax: +33 426 235 916.
E-mail address: brigitte.lemagueresse@inserm.fr (B. Le Magueresse-Battistoni).
[1] Present address: MRC Centre for Reproductive Health, University of Edinburgh, The Queens Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, United Kingdom.

0378-4274/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.toxlet.2011.09.019

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.

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 HAMs 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 interstitial 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 μg/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 μl in a final volume of 0.5 ml 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 manufacturers 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 in 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 s, 55–62 °C (depending on the primers, Table 1) for 10 s, and 72 °C for 20 s. 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