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

3

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7

8 Short title: Impact of TCDD on cultured Leydig cells

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19

20

21 **Abstract:**

22 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related substances are ubiquitous
23 environmental pollutants that exert adverse effects on reproductive processes. In testis,
24 Leydig cells which produce testosterone are under hormonal and local control exerted by
25 cytokines including TNF α . Using mouse Leydig primary cell cultures as a model, we studied
26 the effects of TCDD on the steroidogenic outcome of Leydig cells and the gene expression
27 levels of Ccl5 and Cxcl4, previously shown to be target genes of TCDD in testis. We found
28 that TCDD did not alter the steroidogenic outcome of Leydig cells but that it up-regulated
29 Cxcl4 gene expression levels. TCDD also impacted Ccl5 gene expression when cells had been
30 co-treated with TNF α . TCDD action probably initiated with binding to the aryl hydrocarbon
31 receptor (AhR) present on Leydig cells. TCDD regulated the gene expression levels of AhR
32 (transient down-regulation) and its repressor AhRR and Cyp1b1 (up-regulation). The trophic
33 human chorionic gonadotropin (hCG) hormone did not impact AhR, its repressor AhRR or
34 Cyp1b1 but it opposed the TCDD-enhanced AhRR mRNA levels. Conversely, TNF α
35 stimulated AhR gene expression levels. Collectively, it is suggested that the impact of TCDD
36 on expression of target genes in Leydig cells may operate under the complex network of
37 hormones and cytokines.

38

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

40

41 **1. Introduction**

42 Polycyclic aromatic hydrocarbons (PAHs), as well as halogenated aromatic
43 hydrocarbons (HAHs) such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) and related
44 compounds, are highly persistent environmental pollutants. Dioxins are by-products from
45 incineration processes and pesticide production, and thus they are not intentionally produced.
46 They are considered endocrine disruptors (Diamanti-Kandarakis *et al.* 2009; Hotchkiss *et al.*
47 2008; Lundqvist *et al.* 2006), i.e. chemical substances that interfere with the endocrine
48 systems of humans and wildlife. As such, they are of high concern for human health because
49 endocrine disruptors are suspected to be responsible for apparent changes seen over recent
50 decades, including congenital malformations, cancer and declining sperm counts (Skakkebaek
51 *et al.* 2001; Toppari *et al.* 1996). Dioxins are not genotoxic but cause a broad spectrum of
52 adverse effects including hepatotoxicity, immune system suppression, developmental toxicity,
53 and skin defects. TCDD mediates its toxicity by binding to the aryl hydrocarbon receptor
54 (AhR) and subsequent alteration of the expression of target genes which exhibit dioxin
55 response elements in their promoter moiety including the cytochrome CYP1A1 (Barouki *et al.*
56 2007; Mimura and Fujii-Kuriyama 2003). Apart from phase I and phase II enzymes of the
57 detoxification machinery, microarray studies allowed identifying chemokines as potential new
58 target genes in liver (Boutros *et al.* 2009; Boutros *et al.* 2008) but also in testis (Rebourcet *et al.*
59 2010), suggesting that TCDD exposure may induce inflammatory responses in addition to
60 detoxifying processes. Evidences also accumulated showing that inflammatory cytokines
61 including the tumor necrosis factor α (TNF α) were downstream targets of AhR signalling
62 pathways (Haarmann-Stemmann *et al.* 2009).

63 In the adult testis, Leydig cells, which are fully differentiated cells, produce the
64 male primary steroid hormone, testosterone, in response to their trophic Luteinizing
65 Hormone, LH. In addition to being under hormonal control, Leydig cells are under a local
66 control exerted by autocrine and/or paracrine factors such as cytokines and chemokines
67 (Benahmed 1997; Guazzone *et al.* 2009). Leydig cells are presumed to be somewhat resistant
68 to various types of chemotoxicants due to the insufficiency of CYP1A1 expression and
69 activity as demonstrated elsewhere (Chung *et al.* 2007). The xenobiotic metabolizing enzyme
70 CYP1B1 can also be induced by treatment with AhR agonists (Shimada and Fujii-Kuriyama
71 2004) and in contrast to CYP1A1, CYP1B1 is a predominantly extra-hepatic CYP enzyme.
72 For example, it is highly expressed in adrenals and gonads (Leung *et al.* 2009). However, its
73 regulation by PAHs in steroidogenic tissues is controversial (Deb *et al.* 2010; Mandal *et al.*
74 2001).

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

85

86 **2. Materials and Methods**

87 *2.1. Leydig cell preparation*

88 Swiss male CD-1 mice aged of 8 weeks were purchased from Harlan Laboratories
89 France (Gannat, France). Animals were killed by CO₂ asphyxia. Testes were removed and
90 immediately used for cell preparations. All procedures were performed with the approval of
91 the Regional Committee of Ethics for Animal Experiments.

92 Leydig cells were isolated and cultured in HAM's F12-DMEM (PAA Laboratories,
93 Les Mureaux, France) at 32°C in an humidified atmosphere of 5% CO₂ as previously
94 described (Carreau *et al.* 1988; Mazaud Guittot *et al.* 2008). Briefly, testes were decapsulated
95 and digested with 0.25 mg/ml collagenase at 32°C for 10 min. The digestion procedure was
96 stopped by dilution with fresh medium. Interstitial cells were collected in the supernatant and
97 the pellet containing aggregates of Sertoli and germ cells was discarded. Interstitial cells were
98 purified on a discontinuous Percoll density gradient (layers of 21, 26, 34, 40 and 60%
99 Percoll). The gradient was centrifuged at 800 g for 30 min. The interface between 40 and 60%
100 was collected and washed with medium to remove the Percoll. The presence of 3-β
101 hydroxysteroid dehydrogenase (3beta-HSD) activity was revealed by a histochemical
102 technique described in details elsewhere (Bilinska *et al.* 1997) using the anti-3beta-HSD
103 antibody provided by Dr I. Mason (Reproductive and Developmental Sciences Division,
104 Edinburgh, UK). It was used to determine the purity of Leydig cells (95%). Contamination by
105 interstitial macrophages did not exceed 3%. Cells were resuspended in fresh culture medium
106 supplemented with 2% Fetal Calf Serum (FCS). They were plated in 12-well plates (400,000
107 cells/well). After 24 h, culture media were replaced with serum-free culture media.

108

109 *2.2 Leydig cell treatment*

110 Two days after plating, culture media were renewed and 0.5 ml of fresh serum-free culture
111 media was added per well in the presence or not of different products listed below for the time
112 periods indicated in the text. These products included human chorionic gonadotropin (hCG;
113 100 ng/ml, Organon Schering-Plough, France) to mimic the effect of LH, dibutyryl cyclic
114 AMP (dbcAMP; 0.2 mM, Sigma-Aldrich, France), recombinant Tumor Necrosis Factor alpha
115 (TNF α ; 20 ng/ml; Peprotech France), Interleukin 1 alpha (IL1 α ; 20 ng/ml, Peprotech France),
116 Lipopolysaccharides (LPS; 10 microg/ml; Sigma-Aldrich, France). Cytokines were used at a
117 20 ng/ml concentration to maximally stimulate Leydig cells (Benahmed 1997; Hong *et al.*
118 2004). For 2,3,7,8-tetrachlorodibenzo-p-dioxin (ref ED-901-C; LGC Promochem, Molsheim
119 France), stock solutions were prepared in dimethylsulfoxide (DMSO) and appropriately
120 diluted in culture medium. DMSO volume content per well was 0.04 microl in a final volume
121 of 0.5ml per well. Cell viability was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-
122 (3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] reduction method
123 according to manufacturer's instructions (Promega, Charbonnières, France). Treatments did
124 not alter cell viability which was over 95%. Doses of TCDD higher than 25 nM resulted in
125 loss of cell viability and were not used in the present study.

126

127 *2.3 Testosterone Measurement by Enzyme Immunoassay*

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

131

132 *2.4 RNA extraction, Reverse Transcription and Quantitative PCR (RT-qPCR)*

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

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

155

156 *2.5 Statistical analysis*

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

163

164 **3. Results**

165 *3.1 Steroidogenic outcome of Leydig cells treated with TCDD*

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

173

174 *3.2 Gene expression of AhR, the repressor AhRR and Cyp1b1, and their regulation by TCDD*
175 *and hCG or dbcAMP*

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

190

191 *3.3 Expression of the chemokines Ccl5 and Cxcl4 in primary culture of Leydig cells and their*
192 *regulation by TCDD*

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

200

201 *3.4 Local regulation of the TCDD targeted genes by TNF α*

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

206 not observed at 24 h of treatment. However, TCDD-treated cells did no longer respond to
207 TNF α by enhanced AhR mRNA levels (Fig. 4a). Gene expression levels of Cxcl4 and Ccl5
208 were also investigated. The addition of TNF α , IL1 α and LPS, described as stimulators of
209 chemokines (Graves and Jiang 1995; Nomiyama *et al.* 2010) up-regulated Cxcl4 (Fig. 5a) and
210 Ccl5 (Fig. 5b) gene expression levels in primary culture of Leydig cells. TNF α was a potent
211 stimulator for the two chemokines (4 and 4.4-fold increase for Cxcl4 and Ccl5 respectively,
212 $p < 0.05$) (Fig. 5). By contrast, IL1 α stimulated more Cxcl4 (7-fold increase) than Ccl5 (1.8-
213 fold-increase) gene expression. LPS stimulated more Ccl5 (8-fold increase) than Cxcl4 (2.8-
214 fold increase) gene expression (Fig. 5). Extending data presented in Fig. 3, we observed that
215 TCDD could significantly ($p < 0.05$) oppose the TNF α -induced increase in Ccl5 mRNA levels
216 (Fig. 5b); with no effect on TNF α -induced increase in Cxcl4 mRNA levels (Fig. 5a). A
217 summary of all the observed effects is provided on Table 2.

218

219 **4. Discussion**

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

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

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

239 enhanced the expression of AhRR gene, suggesting negative feedback to limit AhR signalling
240 effect. Indeed, it has been previously shown that AhRR functions as a naturally occurring
241 dominant-negative factor limiting AhR signalling pathway (Abel and Haarmann-Stemmann
242 2010; Mimura *et al.* 1999). Hence, AHRR is an important determinant of tissue specific
243 responsiveness to TCDD, and strong evidences have been reported on an inverse relationship
244 between AHRR expression and sensitivity to induction of xenobiotic-metabolizing enzymes
245 caused by TCDD (Korkalainen *et al.* 2004). The data presented herein i.e., high expression of
246 AhRR and no detectable Cyp1a1 induction are in keeping with these observations.
247 Interestingly, in conditions of increased gene expression levels of StAR following hCG or
248 dbcAMP stimulation, genes encoding AhR and AhRR had unchanged expression levels. We
249 next investigated the combined effects of TCDD with hCG/dbcAMP or TNF α . Indeed, TNF α
250 may originate from the systemic circulation or from the neighbouring interstitial macrophages
251 (Guazzone *et al.*, 2009). We found that addition of hCG which protects testis function against
252 TCDD in adult rats (Lai *et al.* 2005), while not acting directly on AhR or AhRR gene
253 expression levels, attenuated the TCDD-induced stimulation of AhRR. Unlike hCG, TNF α
254 did not reduce TCDD action on the AhRR gene expression levels, and enhanced AhR
255 expression levels. Altogether, these data are in keeping with previous studies showing that
256 gonadotrophins and TNF α exert inverse effects on Leydig cells (Benahmed 1997; Guazzone
257 *et al.* 2009).

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

271 It has been recently demonstrated that TCDD apart from the classical battery of
272 genes of the detoxification machinery, may provoke inflammatory adverse effects
273 (Haarmann-Stemmann *et al.* 2009). For example, it was demonstrated that TCDD could
274 enhance TNF α secretion in a model of cultured adipocytes (Nishiumi *et al.* 2010) as well as
275 in the serum of rats orally treated with TCDD (Ciftci *et al.* 2010). It was also shown that
276 dioxin through binding to AhR could stimulate Ccl5 gene expression in an *in vitro* model of
277 endometriosis (Yu *et al.* 2008; Zhao *et al.* 2002), Ccl1, a chemokine involved in
278 cardiovascular diseases and inflammatory or allergic processes (N'Diaye *et al.* 2006) and Ccl2
279 in multiple organs of mice (Vogel *et al.* 2007). TCDD also inhibited Cxcl12 gene expression
280 and its receptor Cxcr4 in MCF-7 breast cancer cells (Hsu *et al.* 2007). Chemokines are a
281 subset of small chemotactic cytokines produced by both immuno-competent cells and non-
282 immune cells. They are secondary pro-inflammatory mediators that are induced by cytokines
283 such as interleukin-1 or TNF α (Graves and Jiang 1995; Nomiyama *et al.* 2010). We here
284 found that Leydig cells could express Ccl5 and Cxcl4, and that these chemokines responded
285 to stimulation by LPS, IL1 α and TNF α . In addition, depending on the conditions (presence or
286 not of TNF α), these two chemokines were targeted by TCDD in primary cultures of adult
287 mouse Leydig cells, consistently with our previous *in vivo* findings (Rebourcet *et al.*, 2010).

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

303

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

316

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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 | CTTGCACTCGTCTTTGTAC | GACTAGAGCAAGCAATGACAG | 158 | 58 |
| CXCL4 (PF4) | NM_019932.4 | AGCGATGGAGATCTTAGCTGTGTG | GGTCCAGGCAAATTTCTCCCATTC | 160 | 58 |
| AHR | NM_013464.4 | TCATCTGGTTTCTGGCAATGAAT | ATAAGCTGCCCTTGGCATC | 245 | 62 |
| AHRR | NM_009644.2 | TCAGGGGACAAACAGATAGG | CTCAAGTGTACTGGTGTCTC | 220 | 55 |
| CYP1B1 | NM_012940.1 | GCAGCCGCCTTCTGGTAGC | CCACGCGCCTGTCCCTACT | 116 | 60 |
| STAR | NM_000349 | ATGCAGAAGGCCTTGGGCAT | AACACCTTGCCACATCTGG | 113 | 60 |
| CYP11A1 | NM_019779 | ACAAGCTGCCCTTCAAGAAC | TCCTTGATGCTGGCTTTGAG | 223 | 58 |

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

Table 2: Summary of the effects described

| Genes surveyed | Stimuli added in vitro | | |
|-------------------------|------------------------------------------------------------------------------------------|--------------------------------------------------------------|-----------------------|
| | TCDD | hCG/dbcAMP | TNF α |
| Star and Cyp11a1 | no effect | strong up-regulation | not determined |
| AhR | down-regulation at 6 h, not at 24 h; TCDD opposed TNF α -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 TNF α -effect at 24 h | no effect | up-regulation at 24 h |

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476 **FIGURE LEGENDS:**

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

486

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

495

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

503

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

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

511

512 **Figure 5:** Relative expression of Cxcl4 (a) and Ccl5 (b) in primary cultures of Leydig cells
513 recovered from 8-week old mouse testes treated for 24 h with various factors and assessed by
514 RT-qPCR analysis. Factors added included LPS (10 $\mu\text{g/ml}$), $\text{IL1}\alpha$ or TNF α (20 ng/ml), or
515 TCDD (25 nM) or TNF α and TCDD in co-treatment. Cxcl4 (a) and Ccl5 (b) levels were
516 normalized using Rpl19 and are expressed relatively to controls arbitrarily fixed to 1. Values
517 are the mean \pm SEM of $n = 9$ separate experiments. a, $p < 0.05$ versus its respective control. B,
518 $p < 0.05$ versus TNF α –treated cells in (b). Boxes in black correspond to controls and boxes in
519 grey indicate treatment with TCDD.

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Figure 1

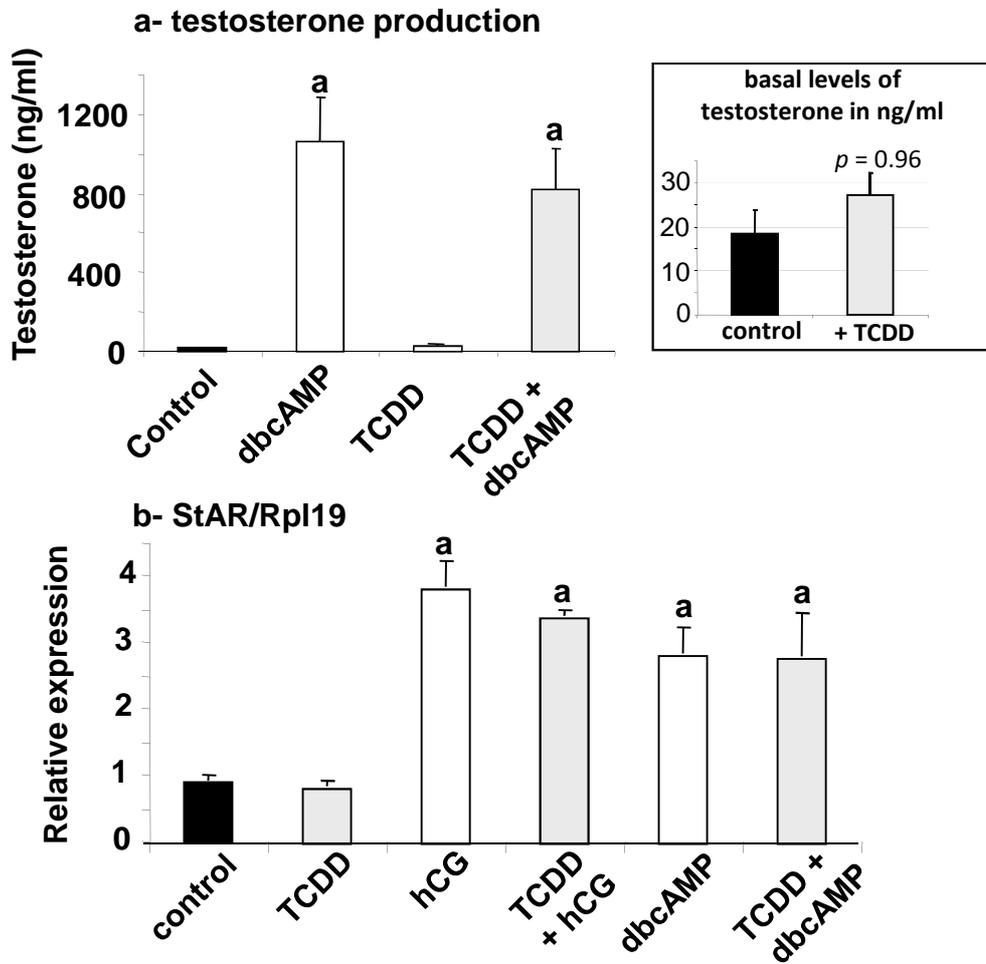


Figure 2

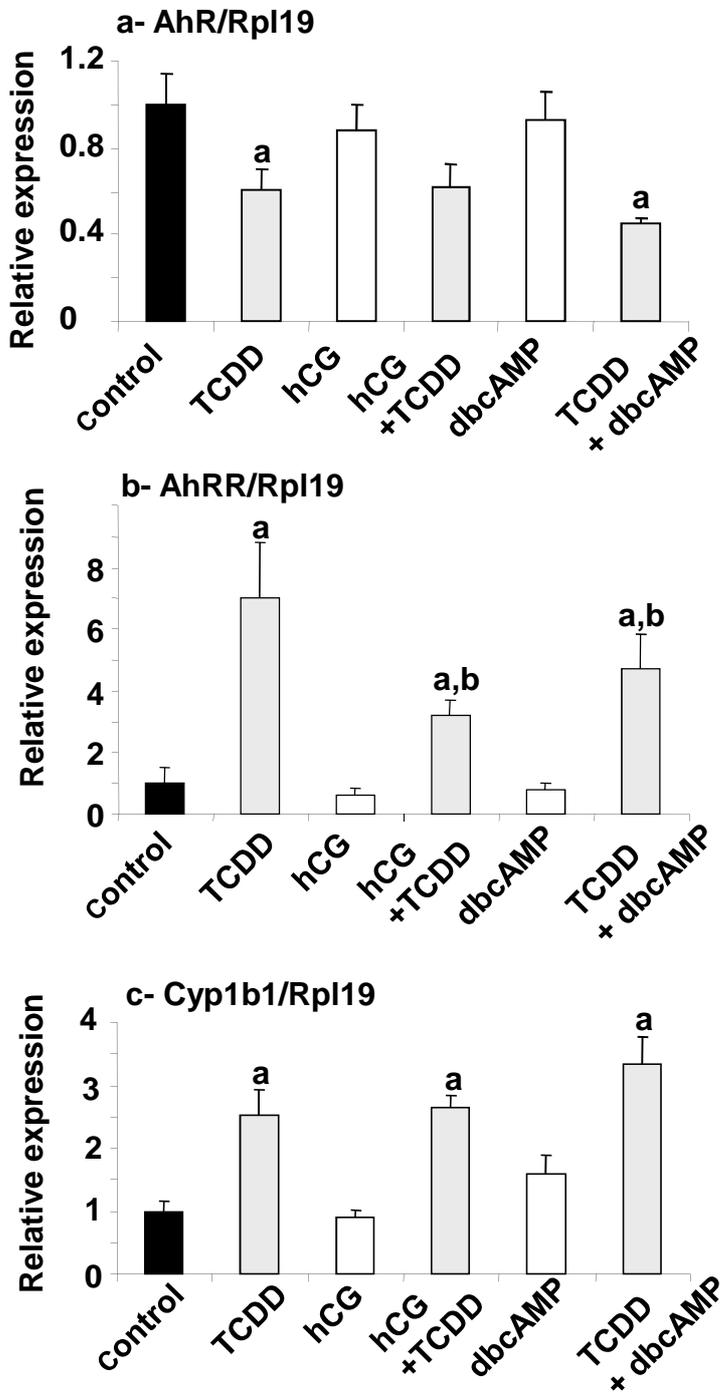


Figure 3

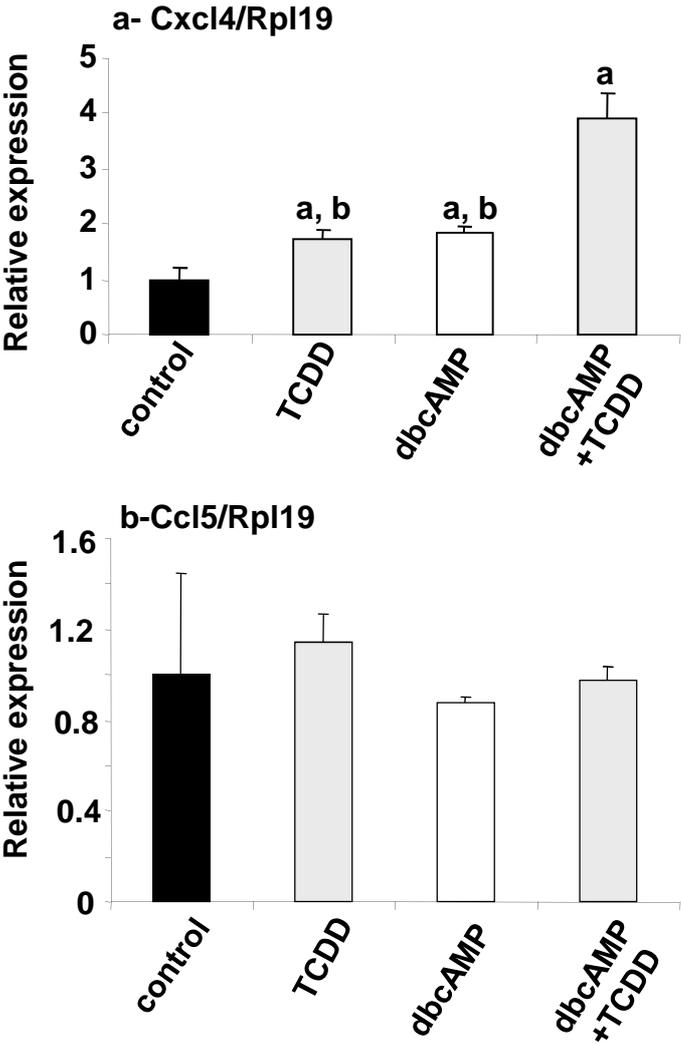


Figure 4

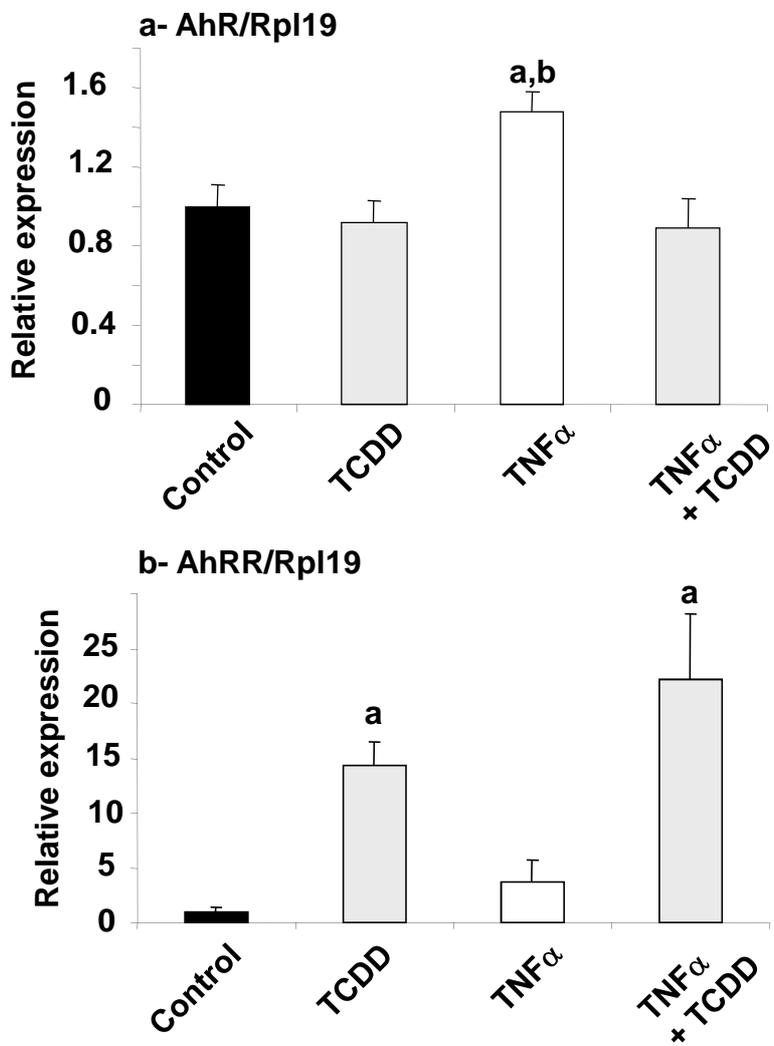


Figure 5

