Effects of PCBs and MeSO$_2$–PCBs on adrenocortical steroidogenesis in H295R human adrenocortical carcinoma cells

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Abstract

Some endocrine disrupting chemicals (EDCs) in the environment have been shown to exert their biological effects through interference with steroidogenesis. In this study, the potential effects of four selected polychlorinated biphenyl (PCB) congeners (PCB101, PCB110, PCB126 and PCB149) as well as several of their environmentally-relevant methylsulfonyl-(MeSO$_2$–) PCB metabolites (3′-MeSO$_2$–CB101, 4′-MeSO$_2$–CB101, 4′-MeSO$_2$–CB110, 3′-MeSO$_2$–CB149 and 4′-MeSO$_2$–CB149) on adrenocortical steroidogenesis were evaluated by in vitro bioassay based on the human adrenocortical carcinoma H295R cell line. The PCBs included in the study represented different structures and potential mechanisms of action. Cells were exposed for 48 h to 10 μM of each PCB congener in the presence or absence of 20% (w/w) of their corresponding MeSO$_2$–PCB metabolite(s). After the chemical treatments, changes in mRNA expression of 11 steroidogenic genes (CYP11A, CYP11B1, CYP11B2, CYP17, CYP19, CYP21, 3β-HSD1, 3β-HSD2, 17β-HSD1, StAR and HMGR) were quantified using molecular beacon-based real-time RT-PCR. Genes coding for enzymes involved in the later or final steps of steroid production (CYP11B1, CYP11B2, CYP19, 3β-HSD1, 3β-HSD2 and 17β-HSD1) were up-regulated to various extents by most PCBs. The greatest transcriptional activations (2.8–29.9-fold) were elicited by PCB110 on CYP11B1, CYP11B2, 3β-HSD2 and CYP19, and PCB149 on CYP11B1, 3β-HSD1 and 17β-HSD1. Increased expression of these steroidogenic genes might ultimately lead to a change in hormonal balance through excessive production of steroid hormones including aldosterone, cortisol and estradiol. In addition, co-treatment with 3′- and 4′-MeSO$_2$–PCB149 resulted in a significant decrease in PCB149-induced 3β-HSD1 and 17β-HSD1 expression. This result indicates that some PCB congeners and their MeSO$_2$-metabolites may affect steroidogenesis via different

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mechanisms. Overall, these findings suggest that PCBs and PCB metabolites can affect regulation of adrenocortical steroidogenesis.

1. Introduction

Polychlorinated biphenyls (PCBs) are one of the most ubiquitous and persistent groups of endocrine disrupting chemicals (EDCs) in the environment. Many of their effects are known to be receptor-mediated, and studies have demonstrated that PCBs can have both agonistic and antagonistic effects on the estrogen and androgen receptors, ER and AR, respectively (reviewed by Ulrich and Stahlmann, 2004), as well as effects that are mediated by the aryl hydrocarbon receptor (AhR). In general, effects of PCB on the ER are dependent on chlorination, with less chlorinated PCBs (<48% chlorine content) such as Aroclor 1248, 1242, etc., acting as estrogen mimics, and the heavily chlorinated PCBs such as Aroclor 1254, 1260, 1262, 1268 and 4465 acting as estrogen antagonists (reviewed by Foster, 1995). Similarly, PCBs have been reported to be AR antagonists, although Aroclor 1254 has also been demonstrated to be a weak AR agonist (reviewed by Ulrich and Stahlmann, 2004).

In terms of AhR activity, some PCB congeners are structurally analogous to polychlorinated-dibenzo-*p*-dioxins while others are not. Congeners that are structurally similar to the dioxins are those that are not chlorinated in the *ortho* positions, which are often referred to as the coplanar congeners as they can achieve a coplanar configuration that allows them to bind with high affinity to the AhR. Alternatively, di-*ortho*-substituted congeners do not bind to the AhR, but act through different mechanisms of toxic action (Giesy and Kannan, 1998; van den Berg et al., 1998). PCB binding to the AhR can also result in antiestrogenic effects due to cross-talk between the AhR and the ER (Safe et al., 1998).

In addition to these receptor-mediated processes, PCBs have also been shown to alter or inhibit hormone production by steroidogenic tissue in reproductive organs (Andric et al., 2001; Gregoraszczuk and Wojtowicz, 2002; Fukuzawa et al., 2003). For example, Aroclor 1248 and 1260 interrupted testicular steroidogenesis in rats through the inhibition of 3β-hydroxysteroid dehydrogenase (3β-HSD), which catalyzes the conversion of pregnenolone to progesterone (Andric et al., 2001). Aroclor 1254 significantly reduced the overall conversion of progesterone to other steroid products in guinea pig adrenal microsomes, largely via the inhibition of 21-hydroxylase activity (CYP 21) (Goldman and Yawetz, 1992). Moreover, PCB126 stimulated basal aldosterone synthesis in human adrenocortical carcinoma H295R cells with concomitant activation of the CYP11B2 gene (Li et al., 2004). In some mammals, PCBs have been found to accumulate in the adrenal gland (Berlin et al., 1975; Brandt, 1977; Biessmann, 1981; Sinjari et al., 1998), an observation which raises the possibility that PCBs can also interfere with steroidogenesis in the adrenal cortex, although this aspect has not been thoroughly studied.

Although PCBs are resistant to biodegradation, some congeners are known to undergo various degrees of in vivo biotransformation (James, 2001). The first step of the multiple biotransformation pathways is the introduction of oxygen via the cytochrome P450 (CYP) enzyme system, producing a hydroxylated metabolite (HO-PCB) or an epoxide. The epoxide can further rearrange to give a HO-PCB or a sulfur-containing metabolite such as mercapturic-, thiol-, methylsulfinyl- or methylsulfonyl- (MeSO$_2$–) PCBs. HO-PCBs are susceptible to conjugation reactions resulting in excretion, but a few are retained in blood as a consequence of their high affinity for the thyroid transport protein transthyretin (TTR) (Chauhan et al., 2000; Letcher et al., 2000, 2004). MeSO$_2$–PCBs, which are more hydrophobic and resistant to further biodegradation than HO-PCBs, accumulate in lipid-containing tissues such as the liver, lung and kidney and typically represent 5–20% of the total PCBs content in these tissues (Brandt and Bergman, 1987; Bergman et al., 1992, 1994; Letcher et al., 1995, 1998, 2000; Stapleton et al., 2001; Larsson et al., 2002; Sandala et al., 2004). The concentrations of total MeSO$_2$–PCBs in tissue samples, which are mainly reported in the fat of marine mammals, are generally in the range of 7–800 ng/g (lipid weight) (Letcher et al., 2000). To our knowledge, there have been no reports of MeSO$_2$–PCB residue levels in adrenal glands.

MeSO$_2$–PCBs, like HO-PCB's, are potential endocrine disruptors. Exposure to MeSO$_2$–PCBs competitively inhibited CYP11B1-dependent corticosterone synthesis in mouse adrenocortical Y1 cells while their parent PCBs did not (Johansson et al., 1998). Environmentally-relevant MeSO$_2$–PCB concentrations have also been shown to inhibit estradiol-mediated induction of vitellogenin in primary carp hepatocytes (Letcher et al., 2002). The existence of persistent PCB metabolites in tissues, and the fact that they differ from their parent compounds in terms of physicochemical properties and

toxicity, necessitates a more comprehensive approach for evaluating the potential toxicological risk of PCB exposure to humans and wildlife.

The H295R cell line is useful for the screening of EDCs that affect adrenocortical function (reviewed by Harvey and Everett, 2003). This cell line has been characterized in detail and the expression of most of the key enzymes and functional proteins in the steroidogenesis pathway has been demonstrated (Gazdar et al., 1990; Staels et al., 1993; Rainey et al., 1994) (Fig. 1). These include CYP11A (side-chain cleavage enzyme), CYP11B1 (11β-hydroxylase), CYP11B2 (aldosterone synthetase), CYP17 (17α-hydroxylase/17,20 lyase), CYP19 (aromatase), CYP21, 3β-HSD, 17β-HSD (17β-hydroxysteroid dehydrogenase), HMGR (3-hydroxy-3-methylglutaryl-CoA reductase), and StAR (steroidogenic acute regulatory protein). Since sequential biotransformations in the steroidogenic pathway require tightly coordinated regulation of these enzymes, alteration in the cellular level or activity of only a single steroidogenic enzyme could result in significant consequences for adrenocortical hormone production as a whole.

In order to monitor these changes at the transcriptional level, sensitive and specific methods have been developed in our laboratories for accurate quantification of expression of 10 steroidogenic genes in H295R cells using real-time RT-PCR (Hilscherova et al., 2004; Zhang et al., 2005). By employing this technique in the current study, mRNA levels of multiple molecular targets in the steroidogenic pathway were quantified in H295R cells upon the in vitro application of four PCBs (PCB101, PCB110, PCB126 and PCB149) and five of their corresponding metabolites (3′-MeSO$_2$–CB101, 4′-MeSO$_2$–CB101, 4′-MeSO$_2$–CB110, 3′-MeSO$_2$–CB149 and 4′-MeSO$_2$–CB149) (Fig. 2). The PCBs selected for analysis represented different structures and potential mechanisms of action: one non-*ortho*-substituted (AhR-active) congener (PCB126); two di-*ortho*-substi-

tuted congeners (non-AhR-active) with substitutions on the same ring (PCB110) and different rings (PCB101); and a congener with three ortho-substituted chlorines (PCB149). In addition, a concentration ratio of PCB-to-MeSO$_2$–PCB of 5:1, which has been measured in the adipose tissues of higher mammals (Letcher et al., 2000), was used in the exposure experiments of this study. The present findings can help elucidate how PCBs and their metabolites can modulate steroidogenesis, which may have implications for human health and environmental risk assessment.

2. Materials and methods

2.1. Chemicals and culture medium

Dulbecco's modified Eagle's medium with Ham's F-12 (DMEM-F12) was obtained from Sigma-Aldrich (St. Louis, MO, USA), ITS + Premix and Nu-Serum from BD Biosciences (San Jose, CA, USA), charcoal dextran-treated FBS from HyClone Laboratory Inc. (Logan, UT, USA), and dimethyl sulfoxide (DMSO; tissue culture grade) from Sigma-Aldrich (St. Louis, MO, USA). PCBs congeners and MeSO$_2$–PCBs were purchased from AccuStandard Inc. (New Haven, CT, USA) and Cambridge Isotope Laboratories (Andover, MA, USA), respectively. IUPAC-based abbreviations for PCB and MeSO$_2$–PCB congener structures have been recently described (Maervoet et al., 2004).

2.2. Cell culture, cytotoxicity and PCB/MeSO$_2$–PCB treatments

Exposure of the cells to chemicals was conducted using a standard protocol as previously described (Zhang et al., 2005). Briefly, human adrenocortical H295R cells (ATCC, Beltsville, MD, USA) were cultivated in DMEM-F12 supplemented with 1 ml/100 ml ITS+Premix and 2.5% BD Nu-Serum in an incubator at 37 °C and 5% CO$_2$. Culture media were refreshed every two to three days.

Cytotoxicity tests were carried out on all test compounds. Standard solutions in DMSO were tested for cytotoxicity towards the H295R cells using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cell suspension (approximately 75 000 cells in 250 μl) was added to each well of 96-Well ViewPlates™ (Packard Instruments, Meriden, CT, USA) and incubated at 37 °C and 5% CO$_2$ for 48 h. When the cells reached 60–80% confluency, the media was changed and replaced with fresh media, and 2.5 μl of the stock standard solutions were added to each well in triplicate. Negative and solvent controls were included on all plates. No significant cytotoxicity was produced by
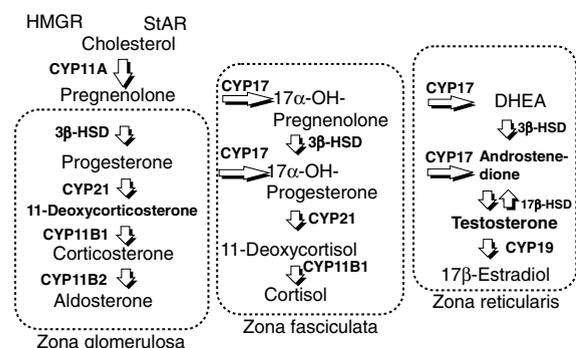

Fig. 1. A schematic of the steroidogenesis pathway showing the substrates involved in steroid production and genes measured in this study.
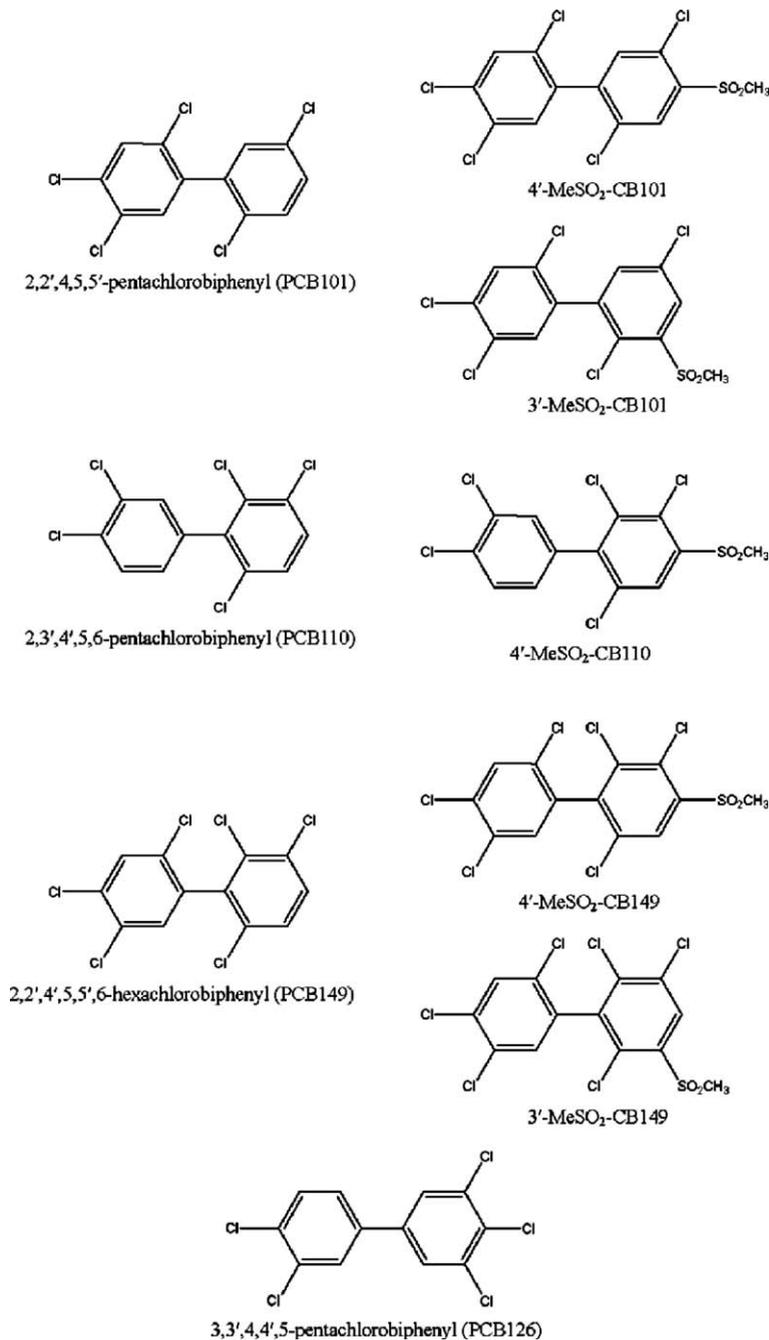
Fig. 2. Structures of the PCBs and $MeSO_2$–PCBs used in this study.

either single PCB congeners or by PCB + 20%-corre-spondent-metabolite at 10 μM concentrations (see Sup-plemental Figure).

Before exposure to PCBs or PCBs + $MeSO_2$–PCBs, cells were transferred to DMEM-F12 supplemented with 1 ml/100 ml ITS + Premix and 2.5% charcoal dextran-treated FBS. The concentrations of PCBs and $MeSO_2$–PCBs used in the exposure were 10 and 2 μM, respec-tively. Six-well tissue culture plates were used in all expo-sure experiments. Cells were dosed with 3 ml of the dosing solution according to the exposure layout. The solvent control group was treated with 3 ml 0.1% DMSO. Exposed cells were incubated for 48 h at 37 °C with 5% CO_2.

Table 1
Sequences of primers and molecular beacons

Gene	Sense primer[a]	Anti-sense primer[a]	Beacon[a]
CYP11A	5′-GAGATGGCACGCAACCTGAAG-3′ (1065)	5′-CTTAGTGTCTCCTTGATGCTGGC-3′ (1201)	5′6-FAM d (CGCGATCTGCCGCGCAGCCAAGACCTCTGATCGCG) DABCYL 3′ (1126)
CYP11B1	5′-GGTTTGCCAGGCTAAGC-3′ (1679)	5′-CAAACTGCCCAGAGGACAG-3′ (1791)	5′6-FAM d (CGCGATCCTGACCTTGTCCCCAGCCCCAGATCGCG) DABCYL 3′ (1729)
CYP11B2	5′-TCCAGGTGTGTTCAGTAGTTCC-3′ (2705)	5′-GAAGCCATCTCTGAGGTCTGTG-3′ (2850)	5′6-FAM d (CGCGATCCCGCAGGTTGCTTTCCCACCCTGATCGCG) DABCYL 3′ (2760)
CYP17	5′-GGCACCAAGACTACAGTGATTG-3′ (246)	5′-AGAGTCAGCGAAGGCGATAC-3′ (392)	5′6-FAM d (CGCGATCAAGGGCAAGGACTTCTCTGGGCGGATCGCG) DABCYL 3′ (306)
CYP19	5′-TTGGAAATGCTGAACCCGATAC-3′ (148)	5′-GCCAGTGAGGAGCAGGAC-3′ (240)	5′6-FAM d (CGCGATCTCGTGCCTGAAGCCATGCCTGGATCGCG) DABCYL 3′ (188)
CYP21	5′-ACCTCAGTTTCTCCTTTATTGC-3′ (1663)	5′-AGAGCCAGGGTCCTTCAC-3′ (1756)	5′6-FAM d (CGCGATCAGGAAGCCTTCTCTGCCAGCGAGATCGCG) DABCYL 3′ (1724)
HMGR	5′-TTCAGGTTCCAATGGCAACAAC-3′ (1702)	5′-GCCACGAGTCATCCCATCTG-3′ (1823)	5′6-FAM d (CGCGATCGCTGGCACCTCCACCAAGACCTAGATCGCG) DABCYL 3′ (1790)
StAR	5′-ATGAGTAAAGTGGTCCCAGATG-3′ (451)	5′-ACCTTGATCTCCTTGACATTGG-3′ (593)	5′6-FAM d (CGCGATCCGGCTGGAGGTCGTGGTGGACGATCGCG) DABCYL 3′ (487)
3β-HSD1	5′-AGCATCTTCTGTTTCCTGGTG-3′ (85)	5′-TCTCCTTCAGCTCCTTCTCCTT-3′ (225)	5′6-FAM d (CGCGATCCCTCCTGCTCCTGTCACAAGGCGATCGCG) DABCYL 3′ (169 for 3B-HSD1; 181 for 3B-HSD1)
3β-HSD2	5′-AGCATCTTCTGTTTCCTGGCA-3′ (96)	5′-TCTCCTTCAGTTCCTTCTCTTC-3′ (237)	
17β-HSD1	5′-TTCATGGAGAAGGTGTTGG-3′ (1545)	5′-AAGACTTGCTTGCTGTGG-3′ (1648)	5′6-FAM d (CGCGATCGACATCCACACCTTCCACCGCTGATCGCG) DABCYL 3′ (1593)
PBGD	5′-CTGGAGGAGTCTGGAGTCTAG-3′ (866)	5′-TGGAATGTTACGAGCAGTGATG-3′ (1002)	5′HEX d (CGCGATCACAAGAGACCATGCAGGCTACCATCGATCGCG) DABCYL 3′ (903)

[a] Numbers in bracket are the nucleotide positions of the primers or beacons in the Genbank gene sequences of the accession numbers, NM_000781 (CYP11A), NM_000497 (CYP11B1), NM_000498 (CYP11B2), M14564 (CYP17), NM_000103 (CYP19), NM_000500 (CYP21), NM_000862 (3β-HSD1), NM_000198 (3β-HSD2), NM_000413 (17β-HSD), NM_000859 (HMGR), NM_000349 (StAR) and X04217 (PBGD).

2.3. RNA isolation and reverse transcription

Total RNA was isolated using the SV Total RNA Isolation kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Before column purification, cells were rinsed twice with ice-cold $1 \times$ PBS followed by lysis with 175 µl SV RNA lysis buffer. The lysate was then passed through a 25 gauge needle to shear genomic DNA. First-strand cDNA synthesis (from 1 µg total RNA) was performed in a 25-µl reaction containing 0.5 µM Oligo(dT)$_{17}$, $1 \times$ first-strand synthesis buffer, 0.5 mM dNTP mix (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 2 U/µl RNaseOUT (Invitrogen, Carlsbad, CA, USA) and 12.5 U/µl N-MLV RT (H-) reverse transcriptase. The reaction was incubated at 42 °C for 50 min and, on completion, was inactivated at 70 °C for 15 min. To digest RNA, 1.25 µl RNase H (Invitrogen) was added and incubated at 37 °C for 30 min. Finally, cDNA was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's protocol.

2.4. Quantitative real-time RT-PCR

The design of gene-specific primers and molecular beacons was based on work by Zhang et al. (2005). The sequences for these primers and probes are shown in Table 1. Real-time (quantitative) RT-PCR was performed using an iCycler system (Bio-Rad Laboratories, Hercules, CA, USA) in sterile 96-well PCR plates (Bio-Rad Laboratories). PCR reaction mixtures contained $1 \times$ PCR buffer II (Applied Biosystems, Foster City, CA, USA), 200 µM dNTP mix (Promega), 5.5 mM MgCl$_2$ (Applied Biosystems), 200 nM molecular beacon, 0.05 U/µl DNA polymerase AmpliTaq Gold (Applied Biosystems), 200 nM or 400 nM sense and anti-sense primers. Genes used with a primer concentration of 200 nM were: StAR, CYP19, 3β-HSD1, CYP11B1 and 17β-HSD1; genes used with a primer concentration of 400 nM were: CYP17, CYP11A, HMGR, CYP21, CYP11B2 and 3β-HSD2. The housekeeping gene prophobilinogen deaminase (PBGD) was used as a reference gene to normalize target gene expression; a PBGD-specific primer was used at a concentration of 200 nM. A final reaction volume of 20 µl was made up with 10 µl diluted cDNA and nuclease-free distilled water (Invitrogen). The PCR reaction mix was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was (1) denaturing for 15 s at 95 °C; (2) annealing for 30 s at 60 °C (all genes except 17β-HSD at 58 °C); (3) extension for 30 s at 72 °C. A total of 50 PCR cycles were used. For each sample, at least triplicate amplifications were performed and the average of all measurements was used in data analysis. The cycle at which the abundance of the accumulated PCR product crosses a specific thresh-

old, the threshold cycle (C_T), was determined for each reaction.

2.5. Calculation of relative gene expression levels

For each experimental sample, the expression level of a target gene was normalized to PBGD, resulting in a mean normalized expression (MNE) value as derived from Eq. (1):

$$\text{MNE} = \frac{(E_{\text{reference}})^{C_T^{\text{reference,mean}}}}{(E_{\text{target}})^{C_T^{\text{target,mean}}}} \tag{1}$$

where E_{target} and $E_{\text{reference}}$ ($=10^{-1/\text{slope}}$) represent the PCR efficiencies determined from the slopes of the standard curves constructed using gene-specific RNA standards of known copy number (Simon, 2003). Final data were calculated as mean normalized steroidogenic gene expression relative to the solvent control, and expressed as:

$$N\text{-fold change} = \text{MNE}_{\text{exp}}/\text{MNE}_{\text{con}} \tag{2}$$

2.6. Statistical analysis

Statistical analyses of gene expression profiles were performed using SigmaStat 3.0.1 (SYSTAT Software Inc., CA, USA). Differences in gene expression were evaluated by ANOVA followed by Tukey's test. Differences with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effects of PCBs on cytochrome P450 enzyme-related gene expression

Expression profiles of CYP11A, CYP11B1, CYP-11B2, CYP17, CYP19 and CYP21 varied in response to the four selected PCBs and three co-treatments with MeSO$_2$ metabolites (Fig. 3). For the purposes of this study, a >25% change in mRNA abundance was considered biologically relevant. CYP11A mRNA expression did not differ appreciably under any of the exposure conditions, whereas induction of CYP17, CYP11B1, CYP11B2, CYP19 and CYP21 varied between the different PCB congener treatments.

CYP17 expression induced by PCB110 was significantly reduced by co-exposure to the congener and its MeSO$_2$ metabolite, but none of the other congeners or metabolites had a relevant effect on gene expression (Fig. 3). Exposure to PCB110, PCB149, PCB126 and PCB101 up-regulated CYP11B1 expression by 3.4-, 2.9-, 1.9- and 1.6-fold, respectively. Co-treatment with 3′- and 4′-MeSO$_2$–CB149 resulted in a significant 1.8-fold decrease of CYP11B1 induction caused by exposure
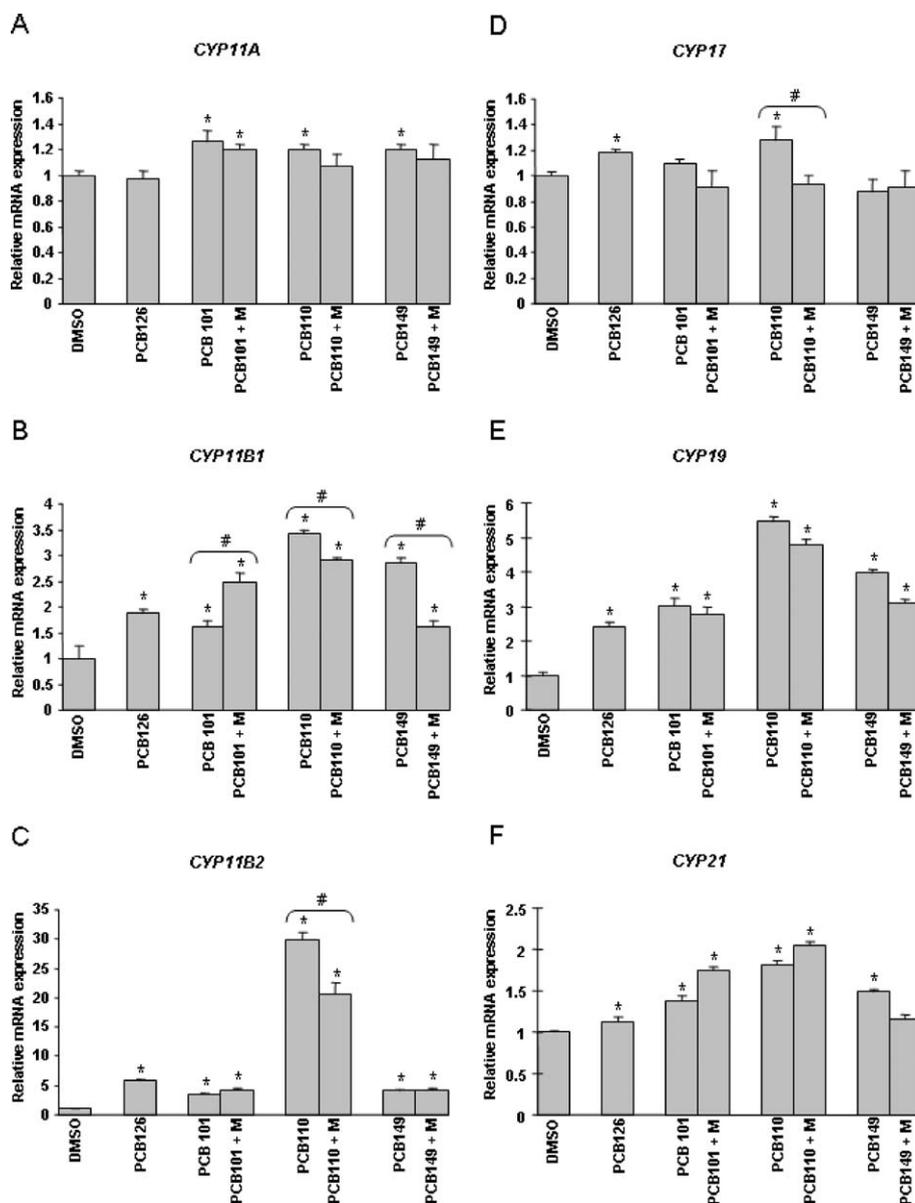
Fig. 3. Effects of selected PCBs and the co-exposure of their MeSO$_2$-metabolites (M) on mRNA expression of steroidogenic cytochrome CYP 450 genes, (A) CYP11A, (B) CYP11B1, (C) CYP11B2, (D) CYP17, (E) CYP19 and (F) CYP21, in H259R cells. Relative abundance of mRNA represents the fold-change in expression with respect to the DMSO solvent control (=1.0). Values presented are the means of three replicate measurements. * $p < 0.05$, statistically different from the DMSO vehicle control; # $p < 0.05$, statistical difference between the parent compound and co-exposure with its metabolite(s).

to PCB149 alone ($p < 0.05$). Likewise, co-treatment with 3′- and 4′-MeSO$_2$–CB101 significantly up-regulated CYP11B1 by 1.6-fold above the induction caused by PCB101 alone ($p < 0.05$). In contrast, co-exposure to PCB110 and its MeSO$_2$ metabolite significantly reduced the up-regulation produced by exposure to PCB110 alone ($p < 0.05$).

CYP11B2 showed the greatest PCB-induced activation among all steroidogenic genes examined. PCB110, PCB126, PCB149 and PCB101 elevated CYP11B2 mRNA expression by 29.9-, 5.8-, 4.0- and 3.4-fold, respectively. Co-treatment with 4′-MeSO$_2$–CB110 resulted in a 1.5-fold decrease in the expression caused by exposure to PCB110 alone. None of the other

congeners or metabolites differed significantly from one another in terms of CYP11B2 gene expression.

The PCB-induced expression pattern of CYP19 was fairly similar to that of CYP11B1. CYP19 transcription was increased by PCB110, PCB149, PCB101 and PCB126 by 5.5-, 4.0-, 3.0- and 2.4-fold, respectively. The effect of $MeSO_2$ metabolites on PCB-induced expression of CYP19 was minimal.

CYP21 expression was least affected by PCBs when compared to the other three PCB-responsive genes; mRNA levels were elevated by 1.8-, 1.5- and 1.4-fold by PCB110, PCB149 and PCB101, respectively. Co-treatment with $MeSO_2$–PCBs did not significantly alter PCB-induced expression of CYP21.

3.2. Effects of PCBs on hydroxysteroid dehydrogenase gene expression

The mRNA expression profiles of the dehydrogenase genes involved in steroidogenesis, 3β-HSD1, 3β-HSD2 and 17β-HSD1, varied among both PCBs and co-treatments with $MeSO_2$ metabolites (Fig. 4). 3β-HSD1 mRNA levels in cells exposed to PCB149 and PCB101

were increased by 3.7- and 1.7-fold, respectively. When exposed jointly to 3′- and 4′-$MeSO_2$–CB149, the stimulatory effect of PCB149 was completely eliminated, bringing 3β-HSD1 mRNA expression to the basal level. 3β-HSD2, an isogene of 3β-HSD1, was up-regulated by all four tested PCBs. PCB110 increased 3β-HSD2 mRNA level by 6.2-fold while PCB149, PCB101 and PCB126 had lesser effects, increasing gene expression by 2.5-, 2.1- and 1.5-fold, respectively. In addition, co-exposure to PCB149 and its metabolites significantly reduced the up-regulation in expression observed with exposure to PCB149 alone ($p < 0.05$). 17β-HSD1 expression was increased by PCB149 by 2.8-fold, but a 5.0-fold decrease in expression was observed when 3′- and 4′-$MeSO_2$–CB149 were co-applied to H295R cells.

3.3. Effects of PCBs on StAR and HMGR gene expression

StAR and HMGR expression responded differently to the selected PCBs and co-treatment with their $MeSO_2$ metabolites (Fig. 5). StAR mRNA levels were decreased by 2.1-fold upon exposure to PCB110. Co-exposure to
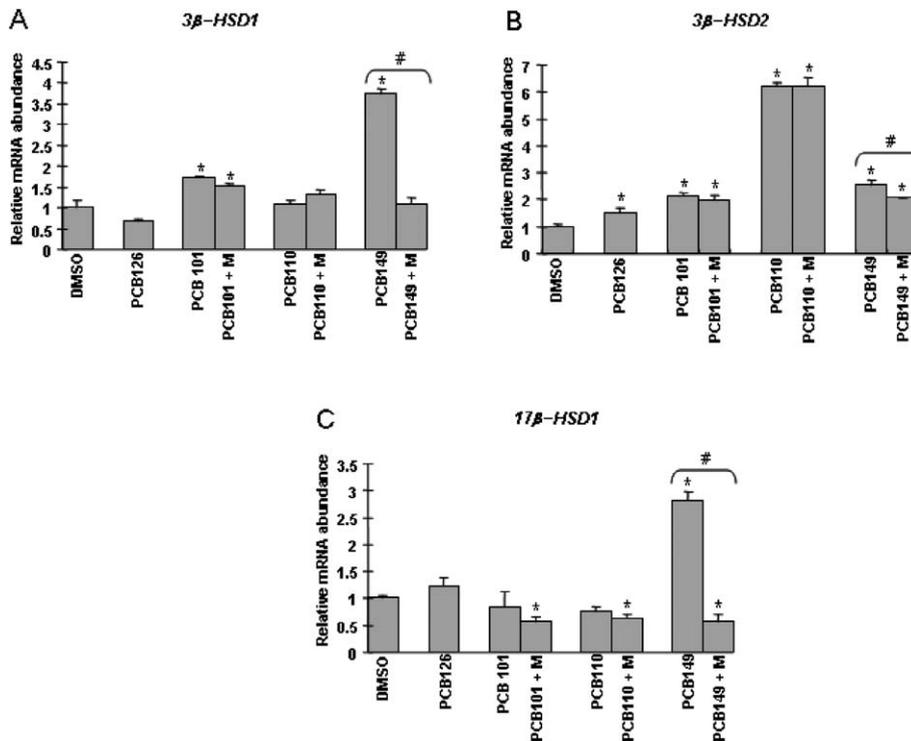

Fig. 4. Effects of selected PCBs and the coexistence of their $MeSO_2$-metabolites (M) on mRNA expression of steroidogenic hydroxysteroid dehydrogenase genes, (A) 3β-HSD1 (B) 3β-HSD2 and (C) 17β-HSD1, in H259R cells. Relative abundance of mRNA represents the fold-change in expression with respect to the DMSO vehicle control (=1.0). Values presented are the means of three replicate measurements. * $p < 0.05$, statistically different from the DMSO vehicle control; # $p < 0.05$, statistical difference between the parent compound and co-exposure with its metabolite(s).
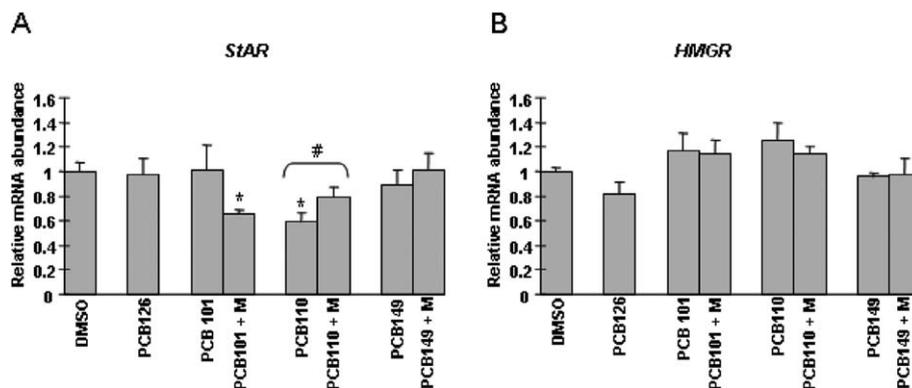
Fig. 5. Effects of selected PCBs and the coexistence of their MeSO$_2$-metabolites (M) on mRNA expression of (A) StAR and (B) HMGR in H259R cells. Relative abundance of mRNA represents the fold-change with respect to the DMSO vehicle control (=1.0). Values presented are the means of three replicate measurements. * $p < 0.05$, statistically different from the DMSO solvent control; # $p < 0.05$, statistical difference between the parent compound and co-exposure with its metabolite(s).

4′-MeSO$_2$–CB110 reduced the inhibitory effect of PCB110 on StAR expression by 1.7-fold. Transcription of HMGR mRNA was not significantly altered by exposure to any of the individual compounds or mixtures.

3.4. Genes affected by PCB110 and PCB149

The results indicate that, compared to the other PCB congeners tested, PCB110 and PCB149 caused the strongest stimulatory effects on the expression of the steroidogenic genes examined in this study. The enzyme-related genes that were affected by PCB110 were CYP11B2, 3β-HSD2 and CYP19, and those affected by PCB149 were 3β-HSD1 and 17β-HSD1.

4. Discussion

Current assessments of the endocrine disrupting properties of chemicals are largely based on the potential for direct interaction of chemicals with hormone receptors such as the estrogen receptor (ER), androgen receptor (AR) and thyroid hormone receptor (ThR). Of particular concern are those compounds that mimic endogenous estrogens, sometimes called xenoestrogens, which are direct-acting estrogen agonists or antagonists that cause effects by binding to the ER. However, there are several other mechanisms of endocrine disruption (Sanderson et al., 1999, 2000, 2001; Hilscherova et al., 2004), including a number of non-receptor mediated processes that may alter endocrine function, emphasizing the inadequacy of purely receptor-based screening strategies for endocrine disruptors. These effects are often exerted indirectly via effects on common signal transduction pathways, or by acting as direct or indirect stimulators or inhibitors of the enzymes involved in the

production, transformation or elimination of steroid hormones (reviewed by Baker, 2001).

The adrenal cortex is often regarded as the most common target for toxicity in the endocrine system (Ribelin, 1984; Colby, 1994) because of (1) the rich blood supply to this small organ, (2) its high lipid content which facilitates the accumulation of lipophilic compounds, and (3) its capacity to synthesize all major classes of steroids (androgens, estrogens, progesterone, as well as glucocorticoids and mineralocorticoids). Despite the importance of adrenocortical function and steroidogenesis to human health, characterizations of the effects of chemicals on adrenocortical function or the molecular pathways of steroidogenesis (other than limited aromatase activity) are surprisingly omitted in current risk assessment programmes.

The H295R cell line is a useful in vitro screening system for both adrenocortical toxicity and alteration in steroidogenesis through the assessment of cortisol production and most of the major enzymes and functional proteins in the steroidogenic pathway (Sanderson et al., 1999). In this study, we demonstrated the effects of four PCBs containing five to six chlorine substituents (PCB126, PCB149, PCB110 and PCB101) and the co-administration of their methylsulfonyl metabolites (except that of PCB126) on the regulation of mRNA expression of 11 steroidogenic genes (six genes encoding cytochrome P450 enzymes: CYP11A, CYP11B1, CYP11B2, CYP17, CYP19 and CYP21; three genes encoding hydroxysteroid dehydrogenases: 3β-HSD1, 3β-HSD2 and 17β-HSD1; two genes involved in cholesterol biosynthesis and transport: HMGR and StAR). With the exception of a recent study on PCB126 by Li et al. (2004), the toxicities of the other three PCBs have not been characterized previously in terms of their abilities to alter steroidogenic gene expression. These four

PCBs were selected because of their high hydrophobicity and tendency to accumulate in lipid-bearing tissues (Letcher et al., 1998, 2000) and the fact that they are frequently found in tissues of wildlife and humans (Wiberg et al., 1998; Letcher et al., 2000; Sandala et al., 2004). To our knowledge, this study is also the first attempt to elucidate the combined effects of PCBs and MeSO$_2$–PCBs on adrenocortical steroidogenesis.

With the exception of CYP11A and CYP17, the examined cytochrome P450 genes (CYP11B1, CYP11B2, CYP19 and CYP21) were up-regulated to different degrees by the tested PCBs. CYP11A catalyzes side-chain cleavage of cholesterol, which is the starting point of steroid synthesis whereas CYP17 controls the initial steps of cortisol biosynthesis by catalyzing the 17α-hydroxylation of pregnenolone (or progesterone) to 17α-hydroxypregnenolone (or 17α-hydroxyprogesterone). Therefore, although CYP11A and CYP17 were not highly induced by exposure to PCBs and their MeSO$_2$ metabolites in this study, it is possible that small changes in their expression may have large effects on steroidogenesis because of the positions that they occupy in the pathway. The marked up-regulation of CYP11B1, CYP11B2 and CYP19 may indicate that the tested PCBs affect the final steps in steroid synthesis.

In the zona glomerulosa, CYP11B2 is responsible for the rate-limiting steps in aldosterone production, involving 11β-hydroxylation of 11-deoxycorticosterone to corticosterone and its subsequent 18-hydroxylation and 18-oxidation. Since aldosterone production is largely controlled by transcription of CYP11B2 (Bassett et al., 2004), increased expression of CYP11B2 should lead to an excess of aldosterone. Elevated aldosterone levels are associated with hypertension, which can cause significant tissue damage to the central nervous system, heart and kidney (Brilla and Weber, 1992; Strandgaard and Paulson, 1994). In this study, the greatest transcriptional activation of CYP11B2 (29.9-fold) was caused by exposure to PCB110, which suggests that this PCB congener may have toxic effects through increased aldosterone synthesis. Although aldosterone levels in the culture medium were not measured in the current study, the correlation between mRNA expression of CYP11B2 and aldosterone production in H295R cells was recently determined by Li et al. (2004), who demonstrated that a 3.3-fold induction of aldosterone by 10 μM PCB126 occurred concomitantly with a 5.1-fold increase in CYP11B2 mRNA expression. Exposure of H295R cells to the same dose of PCB126 in the current study produced a similar magnitude of mRNA induction (5.8-fold), indicating high between-laboratory reproducibility of the H295R bioassay and the real-time RT-PCR technique.

In the zona fasciculata, the final step in the biosynthesis of the glucocorticoid cortisol from 11-deoxycortisol is catalyzed by mitochondrial CYP11B1. Cortisol has numerous metabolic, developmental, immunosuppressive, anti-inflammatory and other functions in the body, including regulating the catabolism of carbohydrates, proteins and lipids. The results of the present study suggest that the four tested PCBs activated CYP11B1 mRNA expression by 1.6–3.4-fold, demonstrating their potential to interfere with the glucocorticoid synthesis pathway in vitro. Again, the strongest transcriptional response resulted from PCB110 treatment.

CYP19 is a crucial steroidogenic enzyme which catalyzes the rate-limiting step in the conversion of androgens to estrogens and its altered expression is a commonly used molecular biomarker of endocrine disruption. In this study, PCB exposure stimulated CYP19 expression in H295R cells by 2.4–5.5-fold. An aryl hydrocarbon-responsive element (AhR/ARNT), which is often associated with exposures to polycyclic aromatic hydrocarbons, has been reported in the zebrafish (*Danio rerio*) CYP19A1 promoter region (Tong and Chung, 2003), which suggests that PCBs may also disrupt endocrine function by directly regulating CYP19 transcription.

PCB-induced CYP19 expression was not altered by the co-administration of MeSO$_2$–PCBs. MeSO$_2$ metabolites are known to be antiestrogenic, and are thought to act by competing with 17β-estradiol for binding sites on the ER (Letcher et al., 2002). The results of this study support a receptor-based, rather than aromatase-based, mechanism of action for MeSO$_2$–PCBs. However, other non-transcriptional regulation of CYP19 by PCB metabolites should not be ignored. A recent study showed that a 24-h exposure to four MeSO$_2$–PCBs, including 4′-MeSO$_2$–CB149, could result in a concentration-dependent decrease of aromatase activity in H295R cells (Heneweer et al., 2004). Therefore, additional studies are required for the comprehensive characterization of the potential effects of PCBs and their metabolites on aromatase function.

3β-HSD is responsible for the oxidation and isomerization of 5-ene-3β-hydroxy steroids to the corresponding 4-ene-3-ketosteroids, an obligate step in the biosynthesis not only of androgens and estrogens but also of mineralocorticoids and glucocorticoids. In humans, two closely related types of 3β-HSD (3β-HSD1 and 3β-HSD2) have been identified and their genes encode deduced proteins of 371 and 372 amino acids, respectively (Luu-The et al., 1989; Rhéaume et al., 1991). 3β-HSD1 is highly expressed in the syncytial trophoblast and in sebaceous glands, and 3β-HSD2 is exclusively expressed in the adrenal cortex and gonads (reviewed by Mason et al., 1997). In vitro kinetic studies have suggested that the catalytic efficiency of 3β-HSD1 with regard to Δ5-steroid substrates is several times greater than that of 3β-HSD2 (Rhéaume et al., 1991). To date, the activity of 3β-HSD1 in human adrenal

glands is unknown (Pang et al., 2003). The results of the present study suggest that the expression patterns of the two isogenes due to PCB exposure are different. Significantly greater expression (>2-fold) of 3β-HSD1 was observed only after exposure to PCB149 whereas comparable up-regulation of 3β-HSD2 was caused by PCB149, PCB110 and PCB101. Differential expression of these two isogenes as a result of PCB exposure suggests that they may be regulated differently and have distinct functional roles in steroidogenesis.

17β-HSD plays a critical role in the regulation of steroid hormones, such as estrogens and androgens, by catalyzing the reduction of 17-ketosteroids or the oxidation of 17β-hydroxysteroids using NAD(P)H or NAD(P)$^+$ as a cofactor. Its involvement in the final step of sex steroid biosynthesis means that the activity of 17β-HSD controls estrogen and androgen concentrations (Darney et al., 1996). In the present study, 17β-HSD1 in H295R cells was up-regulated 2.8-fold by PCB149. This congener also up-regulated 3β-HSD1 (3.7-fold), 3β-HSD2 (2.5-fold) and CYP19 (4.0-fold) by comparable amounts. This concomitant increase in the expression of a sequence of genes that function in estrogen biosynthesis may lead to elevated estrogen production.

StAR transports cholesterol across the mitochondrial membrane for conversion to pregnenolone by CYP11A. Thus, StAR represents an important rate-limiting step in steroidogenesis. StAR mRNA expression was shown to be marginally suppressed by several PCBs used in this study. However, decreased StAR transcription does not necessarily result in reduced cholesterol transport. Recently, work by Sugawara and Fujimoto (2004) demonstrated that the action of StAR is regulated at the translational level and affected by the protein's life-span due to cholesterol-binding, but is not modified by the transcriptional level of StAR. Cholesterol supply to the steroidogenic pathway is critically regulated by the action of HMGR, which controls the rate-limiting step in cholesterol biosynthesis. This study showed that the level of HMGR transcription in H295R cells remained relatively unchanged in response to all PCB treatments, as did CYP11A and CYP17. However, as described above, small changes in these enzyme levels may result in large changes in the steroidogenic pathway. Further research is therefore needed to determine the effects of small-scale changes in gene expression in the early stages of steroidogenesis. Nevertheless, the results of this study indicate that the genes that are most affected by PCBs are likely to be those involved in the final steps of steroid hormone production (e.g. CYP11B2, CYP11B1 and CYP19).

PCB126 was the only congener tested in this study with a co-planar "dioxin-like" structure. As described previously, PCBs with high AhR affinity are known to have antiestrogenic activity through cross-talk with the ER (Safe et al., 1998). In the current study, exposure to PCB126 had no effect on CYP11A, CYP21, 3β-SHD1, 17β-HSD1, StAR or HMGR expression, had a moderate effect on CYP17, CYP19 and 3β-HSD2, and up-regulated CYP11B2 expression by more than 5-fold. These results are largely in agreement with those of a recent study of H295R exposure to PCB126 (Li and Wang, 2005) with the exception of CYP17, which was moderately up-regulated in the current study, but was found to be significantly down-regulated by Li and Wang (2005). The reasons for this disparity are unknown. It is interesting to note that both CYP11A and CYP21 expression was induced by all the other PCB congeners and by some of the metabolites tested, but were unaffected by PCB126. These results support the assertion that PCB126 does not affect steroidogenesis through the AhR (Li and Wang, 2005).

The results of the current study demonstrate that some MeSO$_2$–PCB metabolites can modify the effects of their parent PCBs on the stimulation of steroidogenic gene expression. Generally, co-exposure to the parent compound and its MeSO$_2$ metabolite(s) had an antagonistic effect on the activity of the parent compound. For instance, exposure to 3′- and 4′-MeSO$_2$–CB149 resulted in 3.4- and 5.0-fold decreases in PCB149-induced expression of 3β-HSD1 and 17β-HSD1 expression, respectively. This observation indicates that the methyl-sulfone group may play a role in the inhibition of steroidogenic gene expression. Although the mechanism accounting for this gene inhibition is unclear presently, MeSO$_2$–PCBs have been recently shown to exhibit antiestrogenic activity by competing with E2 for binding to estrogen receptor α (Letcher et al., 2002). This opens up the possibility that MeSO$_2$–PCB antiestrogenicity might increase the unbound level of E2 in vivo, which might in turn attenuate steroidogenesis by feedback inhibition mediated via the suppression of steroidogenic gene expression. However, the effects of whole organism exposure to MeSO$_2$–PCBs are currently unknown.

In summary, PCBs have the potential to cause endocrine disruption, in part by affecting the transcriptional machinery of the steroidogenic genes involved in the final steps of steroid production and, in turn potentially affecting the balance of hormone levels. The antagonistic effect of MeSO$_2$–PCBs on PCB-induced expression of some steroidogenic genes suggests that some PCBs and their metabolites might have opposing effects on steroid hormone biosynthesis. Our findings provide further evidence that the H295R cell line is a useful in vitro model for screening and mechanistic studies of the toxic effects of environmental chemicals on steroidogenesis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2005.08.013.

References

Andric, S.A., Kostic, T.S., Dragisic, S.M., Stojilkovic, S.S., Kovacevic, R.Z., 2001. Acute in vivo and in vitro effects of Aroclors on rat testicular steroidogenesis. In: Robertson, L.W., Hansen, L.G. (Eds.), PCBs: Recent Advances in the Environmental Toxicology and Health Effects. University Press of Kentucky, Lexington, USA, pp. 303–308.

Baker, V.A., 2001. Endocrine disrupters—testing strategies to assess human hazard. Toxicol. In vitro 15, 413–419.

Bassett, M.H., White, P.C., Rainey, W.E., 2004. The regulation of aldosterone synthase expression. Mol. Cell Endocrinol. 217, 67–74.

Bergman, Å., Athanasiadou, M., Bergek, S., Haraguchi, K., Jensen, S., Wehler, E.K., 1992. PCB and PCB methyl sulfones in mink treated with PCB and various PCB fractions. Ambio 21, 570–576.

Bergman, Å., Norstrom, R.J., Haraguchi, K., Kuroki, H., Béland, P., 1994. PCB and DDE methyl sulfones in mammals from Canada and Sweden. Environ. Toxicol. Chem. 13, 121–128.

Berlin, M., Gage, J., Holm, S., 1975. Distribution and metabolism of 2,4,5,2′,5′-pentachlorobiphenyl. Arch. Environ. Health 30, 141–147.

Biessmann, A., 1981. Accumulation of polychlorinated biphenyls in steroidogenic tissue of gonads and adrenals in Japanese Quail. Arch. Environ. Contam. Toxicol. 10, 653–662.

Brandt, I., 1977. Tissue localization of polychlorinated biphenyls—chemical structure related to pattern of distribution. Acta Pharmacol. Toxicol. 40, 1–108.

Brandt, I., Bergman, Å., 1987. PCB methyl sulfones and related compounds: identification of target cells and tissues in different species. Chemosphere 16, 1671–1676.

Brilla, C.G., Weber, K.T., 1992. Mineralocorticoid excess, dietary sodium, and myocardial fibrosis. J. Lab. Clin. Med. 120, 893–901.

Chauhan, K.R., Kodavanti, P.R., McKinney, J.D., 2000. Assessing the role of ortho-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. Toxicol. Appl. Pharmacol. 162, 10–21.

Colby, H.D., 1994. In vitro assessment of adrenocortical toxicity. J. Pharmacol. Toxicol. Methods 32, 1–6.

Darney, K.J., Zirkin, B.R., Ewing, L.L., 1996. Testosterone autoregulation of its biosynthesis in the rat testis: inhibition of 17α-hydroxylase activity. J. Androl. 17, 137–142.

Foster, W.G., 1995. The reproductive toxicology of great lakes contaminants. Environ. Health Perspect. 103 (Suppl. 9), 63–69.

Fukuzawa, N.H., Ohsako, S., Nagano, R., Sakaue, M., Baba, T., Aoki, Y., Tohyama, C., 2003. Effects of 3,3′,4,4′,5-pentachlorobiphenyl, a coplanar polychlorinated biphenyl congener, on cultured neonatal mouse testis. Toxicol. In vitro 17, 259–269.

Gazdar, A.F., Oie, H.K., Shackleton, C.H., Chen, T.R., Triche, T.J., Myers, C.E., Chrousos, G.P., Brennan, M.F., Stein, C.A., La Rocca, R.V., 1990. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Res. 50, 5488–5496.

Giesy, J.P., Kannan, K., 1998. Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. Crit. Rev. Toxicol. 28, 511–569.

Goldman, D., Yawetz, A., 1992. The interference of polychlorinated biphenyls (Aroclor 1254) with membrane regulation of the activities of cytochromes P-450C21 and P-450(17) alpha, lyase in guinea-pig adrenal microsomes. J. Steroid Biochem. Mol. Biol. 42, 37–47.

Gregoraszczuk, E.L., Wojtowicz, A.K., 2002. In vitro exposure of porcine ovarian follicular cells to PCB 153 alters steroid secretion but not their viability—preliminary study. Scientific World Journal 2, 261–267.

Harvey, P.W., Everett, D.J., 2003. The adrenal cortex and steroidogenesis as cellular and molecular targets for toxicity: critical omissions from regulatory endocrine disrupter screening strategies for human health? J. Appl. Toxicol. 23, 81–87.

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X., Sanderson, J.T, Yu, R.M.K., Wu, R.S.S., Giesy, J.P., 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol. Sci. 81, 78–89.

James, M.O., 2001. Polychlorinated biphenyls: metabolism and metabolites. In: Robertson, L.W., Hansen, L.G. (Eds.), PCBs: Recent Advances in the Environmental Toxicology and Health Effects. University Press of Kentucky, Lexington, USA, pp. 35–46.

Johansson, M., Larsson, C., Bergman, A., Lund, B.O., 1998. Structure–activity relationship for inhibition of CYP11B1-dependent glucocorticoid synthesis in Y1 cells by aryl methyl sulfones. Pharmacol. Toxicol. 83, 225–230.

Larsson, C., Ellerichmann, T., Hühnerfuss, H., Bergman, Å., 2002. Chiral PCB methyl sulfones in rat tissues after exposure to technical PCBs. Environ. Sci. Technol. 36, 2833–2838.

Letcher, R.J., Norstrom, R.J., Bergman, Å., 1995. Geographical distribution and identification of methyl sulfone PCB and DDE metabolites in pooled polar bear (Ursus maritimus) adipose tissue from western hemisphere arctic and subarctic regions. Sci. Total Environ. 160–161, 409–420.

Letcher, R.J., Norstrom, R.J., Muir, D.C.G., 1998. Biotransformation versus bioaccumulation: sources of methyl sulfone PCB and 4,4′-DDE metabolites in the polar bear food chain. Environ. Sci. Technol. 32, 1656–1661.

Letcher, R.J., Klasson-Wehler, E., Bergman, Å., 2000. Methyl sulfone and hydroxylated metabolites of polychlorinated biphenyls. In: Paasivirta, J. (Ed.), The Handbook of

Environmental Chemistry: New Types of Persistent Halogenated Compounds, vol. 3. Springer-Verlag, Berlin, pp. 315–359, and references therein.

Letcher, R.J., Lemmen, J.G., van der Burg, B., Brouwer, A., Bergman, A., Giesy, J.P., van den Berg, M., 2002. In vitro antiestrogenic effects of aryl methyl sulfone metabolites of polychlorinated biphenyls and 2,2-bis(4-chlorophenyl)-1,1-dichloroethene on 17β-estradiol-induced gene expression in several bioassay systems. Toxicol. Sci. 69, 362–372.

Letcher, R.J., Chu, S.G., Li, H., 2004. Determination of hydroxylated polychlorinated biphenyls (HO-PCBs) in plasma by high performance liquid chromatography-electrospray-tandem quadrapole mass spectrometry. J. Anal. Toxicol. 29, 209–216.

Li, L.-A., Wang, P.-W., 2005. PCB126 induces differential changes in androgen, cortisol, and aldosterone biosynthesis in human adrenocortical H295R cells. Toxicol. Sci. 85, 530–540.

Li, L.-A., Wang, P.-W., Chang, L.W., 2004. Polychlorinated biphenyl 126 stimulates basal and inducible aldosterone biosynthesis of human adrenocortical H295R cells. Toxicol. App. Pharmacol. 195, 92–102.

Luu-The, V., Lachance, Y., Labrie, C., Leblanc, G., Thomas, J.L., Strickler, R.C., Labrie, F., 1989. Full length cDNA structure and deduced amino acid sequence of human 3β-hydroxy-5-ene steroid dehydrogenase. Mol. Endocrinol. 3, 1310–1312.

Maervoet, J., Covaci, A., Schepens, P., Sandau, C.D., Letcher, R.J., 2004. A reassessment of the nomenclature of polychlorinated biphenyl (PCB) metabolites. Environ. Health Perspect. 112, 291–294.

Mason, J.I., Keeney, D.S., Bird, I.M., Rainey, W.E., Morohashi, K., Leers-Sucheta, S., Melner, M.H., 1997. The regulation of 3β-hydroxysteroid dehydrogenase expression. Steroids 62, 164–168.

Pang, S., Carbunaru, G., Haider, A., Copeland, K.C., Chang, Y.T., Lutfallah, C., Mason, J.I., 2003. Carriers for type II 3β-hydroxysteroid dehydrogenase (HSD3β2) deficiency can only be identified by HSD3β2 genotype study and not by hormone test. Clin. Endocrinol. (Oxf). 58, 323–331.

Rainey, W.E., Bird, I.M., Mason, J.I., 1994. The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. Mol. Cell. Endocrinol. 100, 45–50.

Rhéaume, E., Lachance, Y., Zhao, H.F., Breton, N., Dumont, M., de Launoit, Y., Trudel, C., Luu-The, V., Simard, J., Labrie, F., 1991. Structure and expression of a new complementary DNA encoding the almost exclusive 3β-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase in human adrenals and gonads. Mol. Endocrinol. 5, 1147–1157.

Ribelin, W.E., 1984. The effects of drugs and chemicals upon the structure of the adrenal gland. Fundam. Appl. Toxicol. 4, 105–119.

Safe, S., Wang, F., Porter, W., Duan, R., McDougal, A., 1998. Ah receptor agonists as endocrine disrupters: antiestrogenic activity and mechanisms. Toxicol. Lett. 102–103, 343–347.

Sandala, G.M., Sonne-Hansen, C., Dietz, R., Muir, D.C.G., Valters, K., Bennett, E.R., Born, E.W., Letcher, R.J., 2004. Hydroxylated and methyl sulfone PCB metabolites in adipose and whole blood of polar bear (*Ursus maritimus*) from East Greenland. Sci. Total Environ. 331, 125–141.

Sanderson, J.T., Heneweer, M., Seinen, W., Giesy, J.P., van den Berg, M., 1999. Chloro-*s*-triazine herbicides and certain metabolites induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells. Organohalogen Compd. 42, 5–8.

Sanderson, J.T., Seinen, W., Giesy, J.P., van den Berg, M., 2000. 2-chloro-*s*-triazine herbicides induce aromatase (CYP-19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity. Toxicol. Sci. 54, 121–127.

Sanderson, J.T., Thomas, J., Letcher, R.J., Heneweer, M., Giesy, J.P., Van den Berg, M., 2001. Effects of chloro-*s*-triazine herbicides and metabolites on aromatase (CYP19) activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environ. Health Perspect. 109, 1027–1031.

Simon, P., 2003. Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19, 1439–1440.

Sinjari, T., Wehler, K., Hovander, L., Darnerud, P.O., 1998. Hydroxylated polychlorinated biphenyls: distribution in the pregnant mouse. Xenobiotica 28, 31–40.

Staels, B., Hum, D.W., Miller, W.L., 1993. Regulation of steroidogenesis in NCI-H295 cells: a cellular model of the human fetal adrenal. Mol. Endocrinol. 7, 423–433.

Stapleton, H.M., Letcher, R.J., Baker, J.E., 2001. Metabolism of PCBs by the deepwater sculpin (*Myoxocephalus thompsoni*). Environ. Sci. Technol. 35, 4747–4752.

Strandgaard, S., Paulson, O.B., 1994. Hypertension and human cerebrovascular disease. In: Swales, J.D. (Ed.), Textbook of Hypertension. Blackwell Scientific Publications, Oxford, pp. 690–697.

Sugawara, T., Fujimoto, S., 2004. The potential function of steroid sulphatase activity in steroid production and steroidogenic acute regulatory protein expression. Biochem. J. 380, 153–160.

Tong, S.K., Chung, B.C., 2003. Analysis of zebrafish cyp19 promoters. J. Steroid Biochem. Mol. Biol. 86, 381–386.

Ulrich, B., Stahlmann, R., 2004. Developmental toxicity of polychlorinated biphenyls (PCBs): a systematic review of experimental data. Arch. Toxicol. 78, 252–268.

van den Berg, M., Birnbaum, M.L., Bosveld, B.T.C., Brumström, B., Cook, P., Feely, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., van Leeuwen, F.X.R., Djien Liem, A.K., Nolt, C., Peterson, R.E., Pollinger, L., Safe, S., Schrenk, D., Tillitt, D., Tusklind, M., Younes, M., Waren, F., Zacharewski, T., 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ. Health Perspect. 106, 775–792.

Wiberg, K., Letcher, R.J., Sandau, C., Duffe, J., Norstrom, R.J., Haglund, P., Bidleman, T., 1998. Enantio-selective gas chromatography mass spectrometry of methylsulfonyl PCBs with application to arctic marine mammals. Anal. Chem. 70, 3845–3852.

Zhang, X., Yu, R.M.K., Jones, P.D., Lam, G.K.W., Newsted, J.L., Gracia, T., Hecker, M., Hilscherova, K., Sanderson, J.T., Wu, R.S.S., Giesy, J.P., 2005. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. Environ. Sci. Technol. 39, 2777–2785.