AhR-Agonist-Induced Transcriptional Changes of Genes Involved in Thyroid Function in Primary Porcine Thyrocytes

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The Ah receptor (AhR) is a ligand transcription factor mediating toxic effects of chemicals such as dioxins. The 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and the coplanar polychlorinated biphenyl 126 (PCB 126) are member of the polyhalogenated aromatic hydrocarbons family exerting a variety of toxic effects in a tissue-specific and species-specific manner including thyroid function. In the present study, we aimed to investigate the effects of TCDD (1 and 10 nM) and dioxin-like PCB 126 (306 nM) on the AhR signaling pathway and on the gene expression profiles of key factors involved in thyroid function, including thyroglobulin (TG), thyroid peroxidase (TPO), the sodium iodide symporter (NIS), TSH receptor (TSHR), and cathepsins (Cat B and L), using a primary porcine thyrocyte culture as the experimental model. AhR and ARNT expression was detected both as mRNA and on the protein level. Expression did not vary upon treatment with either TCDD or PCB 126. However, treatment with TCDD and PCB 126 induced an AhR signaling response, as indicated by the expression of the AhR-target gene cytochrome P-450 1A1 (CYP1A1). Both 10 nM TCDD and PCB 126 treatment induced a significant down-regulation in the expression of NIS and cathepsin B without affecting any of the other parameters investigated. In conclusion, these data indicate that (a) thyrocytes are targets of TCDD and TCDD-like compounds and (b) there is evidence for two independent most likely AhR-mediated molecular mechanisms, by which these compounds negatively interfere with thyroid function.

Key Words: tetrachlorodibenzo-p-dioxin (TCDD); polychlorinated biphenyls (PCBs); thyroid, porcine; cathepsin B; sodium iodide symporter (NIS).

INTRODUCTION

Dioxin-like chemicals are ubiquitously present in the environment, and the most potent isomer among the large family of dioxins and related compounds is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Most of the biological effects of TCDD and dioxin-like compounds are thought to be mediated by the activation of the ligand-dependent transcription factor aryl hydrocarbon receptor (AhR) (Schmidt and Bradfield, 1996). Upon TCDD binding, AhR migrates to the nucleus and forms a heterodimer with the AhR nuclear translocator, ARNT. This AhR-ARNT heterodimer binds to the xenobiotic responsive element (XRE) on DNA and activates the transcription of AhR responsive genes (Rowlands and Gustafsson, 1997). Although the gene encoding for cytochrome P-450 1A1 (CYP1A1) has been identified as a highly sensitive marker for AhR nuclear action (Schmidt and Bradfield, 1996; Wilson and Safe, 1998), many genes involved in the AhR-mediated tissue-specific toxicities remain largely unknown.

In addition to reproductive, neurobehavioral, and immunological toxicities, TCDD is also thought to affect thyroid functions (Brouwer et al., 1998; van den Berg et al., 1988). Harbor seals and Beluga whales exposed to a variety of organochlorine compounds, including TCDD, PCBs, and DDT, show a high incidence of goiter (Rolland, 2000). In adult rats, TCDD exposure induced an increase in the volume of thyroid follicular cells followed by hyperplasia of the thyrocytes (Sewall et al., 1995; Wade et al., 2002). Furthermore, exposure to dioxin-like compounds has been demonstrated to affect thyroid endocrine functions by causing hypo-thyroxemia with a reduced blood level of thyroxine (T4) (Meerts et al., 2004; Portier, 2002; Sewall et al., 1995; Weber et al., 1995). AhR-mediated gene activation in primary thyrocytes has not been examined so far. The mechanisms by which TCDD affects the thyroid system and the AhR-dependent genes involved in the impairment of thyroid functions remain to be elucidated.

Thyroid function depends on cycles of synthesis of the prohormone thyroglobulin (TG) and its proteolytic degradation for the maintenance of constant levels of thyroid hormones in the blood of vertebrates (Brix et al., 2001; Herzog, 1984). Thyroglobulin is the major product of thyroid epithelial cells. It is synthesized by thyrocytes and secreted into the lumen of thyroid follicles, where it is stored as the major component of colloid. Thyroid-stimulating hormone (TSH) upregulates TG
synthesis by acting directly on the TG promoter and indirectly through thyroid transcription factors (TTF-1, TTF-2, and Pax 8) (Di Lauro et al., 1995; Dunn et al., 1996). Highly specialized enzymes regulate hormonogenesis in thyroid cells, among which sodium iodide symporter (NIS) and thyroid peroxidase (TPO) are the most important; NIS is a plasma membrane protein that catalyses the active accumulation of iodide in thyroid epithelial cells, a major step in the biosynthesis of thyroid hormones. It couples the inward translocation of Na\(^+\) down its electrochemical gradient to the simultaneous inward translocation of I\(^-\) against its electrochemical gradient (Levy et al., 1998a; Spitzweg and Morris, 2002), using the energy source of Na\(^+/K^+\) ATPase (Ajjan et al., 1998; Carrasco, 1993). Thyroid peroxidase is an integral apical membrane glycoprotein of thyroid follicular cells, which bears a catalytic activity for two substrates and is responsible for both the iodination and the coupling of tyrosine residues in thyroglobulin, leading to thyroid hormone generation (Nunez and Pommier, 1982). Finally, the process of endocytosis of TG-coupled thyroid hormones from the colloid in the thyrocytes must be a strictly regulated process to provide appropriate amounts of thyroid hormones and to avoid excessive hormone release. Recently, it has been demonstrated that partial proteolysis of TG must precede its endocytosis, and this phenomenon is mediated by lysosomal cysteine proteases, such as cathepsin B and L (CatB and CatL). Finally, TG is internalized by thyrocytes for complete degradation and thyroid hormones T\(_4\) and T\(_3\) hormone liberation.

One of the privileged \textit{in vitro} models to analyze different aspects of thyroid cell biology, especially iodine metabolism and thyroid hormone biosynthesis, is the porcine thyroid cell primary culture system. Indeed, freshly isolated porcine thyrocytes are able to reconstitute functional thyroid follicles in short-term culture (Levy et al., 1998b; Munari-Silem et al., 1990), allowing investigations under conditions rather close to the \textit{in vivo} physiological context. Furthermore, although no data are available on the sensitivity of porcine thyroid to dioxin-like compounds, several studies indicate that other endocrine organs, such as the gonads, represent a target for organochlorine AhR-ligands in this species (Gregoraszcuk, 2002; Wojtowicz et al., 2004). In the present study, we have employed isolated primary porcine thyrocytes as an \textit{in vitro} model to investigate AhR-mediated gene responses upon exposure to TCDD and to the dioxin-like coplanar AhR ligand PCB 126. To accomplish this, we used CYP1A1 gene activity as an indicator of the functional activation of the AhR in pig thyrocytes. Furthermore, we determined the effect of TCDD and PCB 126 on the gene expression profiles of key factors involved in thyroid function, including TG, TPO, NIS, TSH receptor (TSHR), and cathepsins.

**MATERIALS AND METHODS**

**Chemicals.** 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and 3,3’,4,4’,5-pentachlorobiphenyl (PCB 126) were purchased from Okometric (Bayreuth, Germany). All chemicals were dissolved in dimethylsulfoxide (DMSO) and added to the cells at a final concentration of 1 nM and 10 nM for TCDD, and 306 nM for PCB 126.

**Collection of primary pig thyrocytes.** Thyroid glands were obtained from young pigs from Germany. Thyroid glands were obtained from 10-week-old pigs (German Landrace × Large White) used in ethically approved training programs of the Faculty of Medicine. The glands were transported on ice to the laboratory, and connective tissue was removed. The thyroid tissue was then cut into ~2 mm pieces with razor blades. After repeated washing with Hank’s buffered salt solution (HBSS), the thyroid fragments were pelleted at 100 x g for 2 min. The pellets were resuspended in HBSS containing 0.2% collagenase (Biochrom AG, Berlin, Germany) and incubated for 30 min at 37°C on a heattable magnetic stirrer. The suspension was dissociated by repeated pipetting, filtered through a sieve, and pelleted at 100 g for 1 min. The pellet was washed six times in DMEM supplemented with gentamicin (0.5%). The final pellet was resuspended in DMEM containing 0.5% gentamicin and 10% FCS, plated into 6-well cell culture dishes and incubated at 37°C in a humidified CO\(_2\)-incubator. Thyrocytes were grown without further passage for 24 h prior to the experiments. Incubations with TCDD, PCB 126, and DMSO-control were performed on subconfluent primary porcine thyrocytes for 24 h at 37°C in a humidified CO\(_2\)-incubator.

**Isolation of RNA and semi-quantitative RT-PCR.** Primary cells were lysed in Trizol reagent (Invitrogen, Karlsruhe, Germany), total RNA was extracted following the manufacturer’s protocol and RNA dissolved in water was stored at −80°C until use. First strand cDNA synthesis was prepared from total RNA (1 µg) by reverse transcription using Moloney murine leukemia virus (M-MLV) reverse transcriptase and the random hexamer primers (both from Applied Biosystems, Foster City, CA, USA). Based on mRNA coding sequences available from the European Molecular Biology Laboratory (EMBL) databank, porcine-specific primer pairs for polymerase chain reaction (PCR) were designed as follows: \textbf{β-Actin} (accession number U07786) sense primer: GTGCAGGACATCAAGGGAAAG, antisense primer: CGATCCACACGGAG-TATCTTGGC; \textbf{AhR} (accession number AY078127) sense primer: AGAGTG-GGCATGATAATGTGTC, antisense primer: GCCCTAGGTGTTCTCTAATGTTT; \textbf{NIS} (accession number NM173993) sense primer: CAGCAAAAGGGAATTGGATA, antisense primer: GCTGGCAATGGTTCAGGAGG; \textbf{CYP1A1} (accession number AB052254) sense primer: TTGGCTCAGACCCAGCTTTCC, antisense primer: TGTGTCAAAACCCAGCTCTCAAG; \textbf{TSHR} (accession number AY429111) sense primer: ACACGGGGCTGACTCTCTTACAT, antisense primer: TTTATGTTGCTGTTAGGAGAG; \textbf{TPO} (accession number X40645) sense primer: ATCTGAGACCTGCGCCACCTC, antisense primer: ACACGGCCTGACACTCTTGAGG; \textbf{TGF-\beta} (accession number AB068312) sense primer: CAGCGGCTCTCCTACCCTCTTCT, antisense primer: TGCTCTTCAGACCTCGG; \textbf{NIS} (accession number U60282) sense primer: CGGCCCTGGCCTATCCTGAAACAAAG, antisense primer: CAGCAGTGGAGCAGAGCCACAG; \textbf{CatB} (accession number NM174031) sense primer: AAGCCTGGCTCTTCCCTCTTG, antisense primer: TITCCAGGTTGTCGCCACAG.

**Semiquantitative PCR.** For semiquantitative RT-PCR, the different RNA samples were normalized for RNA input by co-amplification of \textbf{β-Actin} transcripts as an internal standard. The amplification reaction was stopped within the exponential amplification phase. Amplifications were performed on 2 µl first strand cDNA in a 30-µl final volume containing 0.2 µM of the primer combinations (see above), 1 U Taq polymerase, 1.5 mM Mg\(_2\)Cl\(_2\), 1 x PCR buffer (all reagents from Life Technologies), and 0.2 nM dNTPs (MBI Fermentas, St. Leon-Rot, Germany). Polymerase chain reaction cycles comprised a 30-s denaturation step at 94°C, 30-s annealing at 55°C–60°C, and a 45-s elongation step at 72°C, followed by a final extension period for 10 min at 72°C. All samples were amplified with an intron-exon spanning primer pair to avoid possible genomic DNA amplification, and a water control was included in all PCR reactions to detect contamination. A volume of 20 µl per PCR reaction was subjected to electrophoresis on a 1.5 % agarose gel in Tris-acetate-ethylene diamine tetraacetic acid (EDTA; TAE) buffer containing 0.2 µg/ml ethidium bromide, and ampiclons were visualized at 312 nm. Gel images were digitized with a CCD camera, and the intensity of each band was quantified by.
densitometric analysis using a computer-assisted image analysis system (BioProfil, LTF-software, Wasserburg, Germany). All experiments were replicated at least three times, and the mean relative amounts of the mRNA of interest were calculated as a percentage of the intensity of the β-actin band for each amplicon.

**Western Blot analysis.** Cells were lysed in 2× Laemmli sample buffer (Laemmli, 1970) in the presence of protease inhibitors (Sigma, Munich, Germany—cat no. P2714). Total extracted proteins were separated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Braunschweig, Germany). Nonspecific binding sites were saturated with 5% nonfat dry milk, and membranes were incubated with various antibodies including a rabbit polyclonal antibody against CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal antibody to AhR (MA1-514, clone RPT1; Dianova, Hamburg, Germany), all diluted 1:250 in 5% nonfat dry milk. Peroxidase-conjugated secondary antibody was from Pierce (Bonn, Germany), and immune complexes were detected with the enhanced chemiluminescence kit West Pico ECL (Pierce) following the manufacturer’s protocol. Finally, peak areas of the immunobands were determined with the ChemiDoc documentation system (Lab-Works software 4.5—LTF).

**Statistical analysis.** Data are presented as mean percentages (± SEM) of a minimum of three independent experiments. Statistical analysis was performed using analysis of variance (ANOVA) followed by the Duncan test. In all cases the criterion for significance was set at $p \leq 0.05$.

**RESULTS**

Isolated porcine thyrocytes exposed in vitro to TCDD or PCB 126 did not show a change in AhR and ARNT gene expression (Fig. 1a) and in AhR immunoreactive protein production in total cell lysates (Fig. 1b). However, porcine thyrocytes responded to TCDD and PCB 126 with an increased expression of CYP1A1. A marked increase in CYP1A1 gene activity was detected upon exposure to TCDD doses as low as 1 nM (Fig. 2a). By contrast, increased expression of CYP1A1 protein was only detected in thyrocytes that had been exposed to 10 nM TCDD, whereas those cultures treated with 1 nM TCDD or DMSO solvent were devoid of CYP1A1 immunobands (Fig. 2b). Similar to TCDD, thyrocytes responded to the dioxin-like AhR-agonist PCB 126 with a significant increase in the expression of CYP1A1, both at mRNA and protein level (Fig. 2a, b). Thus, isolated pig thyrocytes contained a functional AhR-ARNT transcriptional system resulting in the AhR agonist-dependent upregulation of CYP1A1.

To study the effects TCDD and PCB 126 on thyroid function, we performed a transcriptional analysis of genes encoding for proteins relevant for thyroid endocrine functions. Treatment of cultured porcine thyrocytes with TCDD (1 nM and 10 nM) and PCB 126 (306 nM) had no effect on the expression of genes for the thyroid-stimulating hormone receptor (TSHR), thyroglobulin (TG), and thyroid peroxidase (TPO) (Fig. 3). By contrast, we observed a significant downregulation in Na$^+/I^-$ symporter (NIS) gene activity at 10 nM TCDD or 306 nM PCB 126, suggesting a potential involvement of AhR agonists in affecting iodine uptake by these primary porcine thyrocytes.

In exposed thyrocytes, expression analysis of the lysosomal proteolytic cathepsins, known to be involved in TG catabolism, revealed a significant downregulation in CatB mRNA upon exposure to 10 nM TCDD or 306 nM PCB 126. By contrast, CatB gene activity was unchanged when primary pig thyrocytes were exposed to 1 nM TCDD or DMSO controls (Fig. 4).

**DISCUSSION**

In the present study we have, for the first time, demonstrated a TCDD- and dioxin-like PCB-induced effect on the transcriptional regulation of key enzymes involved in thyroid hormone...
thyroid cells of the pig thyroid gland. TCDD and PCBs are known to alter thyroid morphology and disrupt thyroid function leading to hyperplasia of the thyroid gland and modulation of the hypothalamic-pituitary-thyroid (HPT) axis (Nishimura et al., 2003; Sewall et al., 1995). TCDD- and PCB-induced endocrine disruption of negative feedback mechanisms within the HPT axis appears to be due, at least in part, to the hepatic induction of the microsomal phase II enzyme UDP-glucuronosyltransferase resulting in enhanced glucuronidation and excretion of T₄-glucuronide (Van Birgelen et al., 1995). However, whether dioxin-like compounds can directly affect the synthesis and transport of thyroid hormone in the thyroid follicular cells remained to be elucidated.

We investigated the effect of TCDD and PCB on the transcriptional regulation of four genes related to main endocrine functions of the thyroid gland, namely Na⁺/I⁻ symporter (NIS), thyroid peroxidase, thyroglobulin, and TSH receptor. While the latter three genes were unaffected by TCDD and PCB 126, both AhR agonists significantly decreased the expression of NIS in the cultured porcine thyrocytes. In follicular thyrocytes, NIS couples the influx of Na⁺ along its electrochemical gradient to the simultaneous inward translocation of I⁻ against its electrochemical gradient in an ATP-dependent process (Levy et al., 1998a; Spitzweg and Morris, 2002). A specific decrease of NIS gene activity has been reported in thyroid adenomas, and even more extensive downregulation of NIS and TPO has been shown in thyroid carcinoma, suggesting that the low expression of NIS may represent an early response during the thyrocyte transformation pathway (for review see Filetti et al. [1999]). In rodents, oral administration of TCDD resulted in a dose-dependent formation of follicular thyroid adenoma (National Toxicology Program, 1982), and thyroid

FIG. 2. (a) Expression of CYP1A1 mRNA in porcine thyrocytes after culture in the presence or absence of TCDD (1 and 10 nM, respectively) or 306 nM PCB 126. CYP1A1 and β-actin mRNAs were detected using specific RT-PCR in the same samples of thyrocytes harvested at 24 h of culture. The CYP1A1/β-actin densitometric ratio is shown (mean ± SEM). (b) CYP1A1 protein was detected by Western blot analysis. Lanes 1–4: solubilized extracts of porcine thyrocytes harvested at 24 h of culture in the presence or absence of TCDD (1 and 10 nM, respectively) or 306 nM PCB 126.

FIG. 3. Expression of TG, TSHR, NIS, and TPO mRNA in porcine thyrocytes after culture in the presence or absence of TCDD (1 and 10 nM, respectively) or 306 nM PCB 126. Thyroglobulin, TSHR, NIS, TPO, and β-actin mRNAs were detected using specific RT-PCR in the same samples of thyrocytes harvested at 24 h of culture. The gene/β-actin densitometric ratio is shown (mean ± SEM).
adenoma was frequently observed in Beluga whales with high PCB tissues concentrations (Mikaelian et al., 2003; Rolland, 2000). The specific downregulation of NIS gene expression in primary porcine thyrocytes in response to TCDD and PCB 126 may provide first evidence for a functional mechanism by which dioxin-like compounds negatively interfere with iodine uptake by the thyroid, thus contributing to adenoma formation.

After exocytosis, thyroglobulin (TG) is stored within the extracellular lumen of thyroid follicles in a covalently cross-linked form of globules up to 120 µm in diameter. The degradation of thyroglobulin and the liberation of T4 and T3 constitute important steps in thyroid endocrine function, and lysosomal cysteine proteinases of the cathepsin family, among them cathepsin B and L, are known to mediate proteolytic processing of thyroglobulin (Brix et al., 1996; Friedrichs et al., 2003). Primary porcine thyrocytes expressed CatB, whereas CatL expression could not be demonstrated with any confidence. Both, TCDD and PCB 126, caused a significant downregulation of cathepsin B gene activity. Cycles of TG deposition and TG proteolysis regulate the size of the luminal content of thyroid follicles (Brix et al., 2001; Linke et al., 2002). CatB-deficient mice were shown to display extreme accumulation of TG accompanied by increased size of thyroid follicles and enlarged thyroid glands (Friedrichs et al., 2003), likely as a result of impaired proteolysis of TG from the lumen.

It is noteworthy that acute in vivo exposure to TCDD induces thyroid hyperplasia in rats, accompanied by an increased number of abnormally shaped follicles (Nishimura et al., 2003). Furthermore, in vivo exposure to PCBs in birds is characterized by augmented thyroid weight and increased thyroidal colloid (Saita et al., 2004). According to these data, the downregulation of the CatB gene observed in the present study may provide a first indication that dioxin-like compounds are involved in reduced solubilization of TG, resulting in histopathological changes in the thyroid.

Finally, the fact that TCDD and coplanar PCB 126 caused similar attenuation of NIS and CatB gene activity suggests that the arylhydrocarbon receptor mediates these genomic effects in porcine thyrocytes. This hypothesis is further supported by the observation that in vivo administration of TCDD does not induce any negative effects on thyroid hormone homeostasis in AhR⁻/⁻ mice (Nishimura et al., 2005).

Our present findings in porcine thyrocytes primary cultures would suggest two independent, most likely AhR-mediated, molecular mechanisms induced by TCDD and TCDD-like compounds, which may explain the enlarged thyroid glands.
observed in TCDD-treated animals in vivo. The downregulation of NIS would restrict iodine intake by the thyroid, causing compensatory cellular hypertrophy of thyrocytes while the decrease in CatB could lead to attenuated CatB proteolytic cleavage and accumulation of TG in thyroid follicles (Fig. 5). This study has focused on the acute and short-term effects of TCDD on primary porcine thyrocytes. Additional work is needed to determine the molecular effects of chronic exposure to dioxin-like compounds in thyrocytes.

In summary, we have demonstrated a functional AhR signaling in primary porcine thyroid follicular cells. Both TCDD and coplanar PCB 126 significantly downregulated NIS and cathepsin B gene activity in porcine thyrocyte primary cultures, suggesting novel molecular mechanisms for AhR-mediated actions in thyrocytes.

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