Environmental Toxicology

POLYCHLORINATED BIPHENYL 126 AFFECTS EXPRESSION OF GENES INVOLVED IN STRESS–IMMUNE INTERACTION IN PRIMARY CULTURES OF RAINBOW TROUT ANTERIOR KIDNEY CELLS

ELGAR SUSANNE QUABIUS,*† GUIDO KRUPP,‡ and CHRISTOPHER J. SECOMBES†
†Scottish Fish Immunology Research Centre, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, Scotland, United Kingdom
‡artus Königstrasse 4a, 227667 Hamburg, Germany
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Abstract—Stress and immune function are linked in all vertebrates, including teleost fish. Polychlorinated biphenyls (PCBs) are immunotoxic and impair the ability of fish to respond to additional stressors. In this study, we investigated the effects of PCB126 on stress and immune function and the interaction of these systems in fish using primary cultures of rainbow trout anterior kidney cells as a model. Gene expression levels of cytochrome P4501A (CYP1A), interleukin-1β (IL-1β), and glucocorticoid receptor (GR) were measured by real-time quantitative polymerase chain reaction. These genes play important roles in detoxification and immune and stress homeostasis, respectively. Incubation with PCB126 led to increased IL-1β expression between 30 min and 2 h of exposure, with expression back to basal levels after 6 h. Lipopolysaccharide (LPS) incubation evoked normal IL-1β responses after 2 and 24 h PCB incubation. Gene expression levels of GR and CYP1A increased in a time- and dose-dependent manner, reaching a plateau after 12 h of incubation. Preincubation with cortisol resulted in decreased IL-1β expression, increased expression of CYP1A and GR, and was accompanied by an abolished PCB responsiveness after more than 4 h of cortisol incubation. We conclude that PCB126 exposure is not "stressful," as increased cortisol levels would result in depressed IL-1β expression. Incubation with PCB126 evokes a transient stimulation rather than permanent damage of the immune system, as LPS stimulation resulted in increased IL-1β expression after PCB incubation. Prolonged cortisol preincubation, resembling a chronic stress paradigm, negatively affects the immune responsiveness of the cells as well as their capacity for toxicant metabolism.

Keywords—Teleost fish, Teleost anterior kidney, Primary cell culture, Stress–immune interaction, Polychlorinated biphenyl 126

INTRODUCTION

Aquatic vertebrates, such as teleost fish, are good indicator species to assess pollutants in the aqueous environment, as seen with endocrine disrupters [1]. Not only is the endocrine system affected by pollutants, but many aspects of the immune system also appear to be particularly sensitive [2]. The immunosuppressive effects described in previous studies [2] range from pathological effects to effects on lymphocyte numbers and their proliferation capacity and may in part be induced by stress effects on the immune system, directly inhibiting lymphocyte responses or indirectly affecting the release or functioning of signaling molecules within the immune system (e.g., cytokines) [3]. While the role of cytokines in initiating and regulating immune responses is clear, they also interact with neuroendocrine pathways, particularly the hypothalamus–pituitary–gonadal (HPG) axis [4]. For example, secretion of cytokines such as interleukin-1β (IL-1β) during infection will act on the hypothalamus to induce release of corticotropin-releasing factor, resulting in activation of the pituitary–interrenal part of the HPI axis and ultimately in an increase in cortisol production and secretion from the head kidney interrenal cells (reviewed by Wendelaar Bonga [5]). On the other hand, factors modulating cytokine expression can have profound effects on the ability to resist infection, with the potential to negatively affect fish populations. For example, it is known from the teleost literature that corticosteroids released during stress responses can lead to suppression of immune responses [6].

Polychlorinated biphenyls (PCBs) are particularly interesting environmental contaminants to study for their effects on the fish immune system. They are highly toxic environmental pollutants that are found in aquatic systems throughout the world [7] and have a known ability to disrupt endocrine pathways, involving the hypothalamus–pituitary–gonadal, hypothalamus–pituitary–thyroid, and HPI axis [8–10]. In fish, it has been known for many years that PCB exposure affects disease resistance [11]. More recently, studies have shown that a number of cellular immune parameters are modulated by PCB exposure [2]. Particularly interesting is the fact that PCBs are known to affect cytokine secretion in mammals [12], with cytokine disruption likely to compromise disease resistance.

Studies on the effects of PCB and related organic pollutants have focused on either the stress response, the immune system, or detoxifying processes [13]. For example, primary cultures of head kidney cells isolated from rainbow trout (Oncorhynchus mykiss) treated with organic toxicants showed decreased cortisol responses to stimulation with adrenocorticoid hormone, indicating an impairment of the HPI axis. In another study [14], it was shown that 3-methylcholanthrene induced 7-ethoxyresorufln-O-deethylase (EROD) activities in head kidney of carp (Cyprinus carpio) and that this response could be modulated by cortisol incubation. Interestingly, similar effects of cortisol on EROD activities could be demonstrated in primary cultures of trout hepatocytes [15]. In a more recent study [16], the effects of confinement on EROD activities in a variety of tissues were investigated, confirming the modifying effects
of cortisol on EROD activities under physiological conditions. Furthermore, it could be demonstrated that PCB126 does accumulate in the head kidney of tilapia (Oreochromis mossambicus) exposed orally to this substance [17].

The aims of the study presented were to investigate the interplay of PCB126, the stress axis, and immune responses using primary cultures of rainbow trout anterior kidney cells as a model. In the present study, immune and stress function were assessed by measuring gene expression levels of IL-1β and glucocorticoid receptor (GR). Cytochrome P4501A (CYP1A) expression level was measured to assess detoxification processes. These genes were chosen because they play an important role in the regulation of the immune system, the stress response, and the response to toxicant exposure, respectively [3,18,19]. In addition, these genes are accepted for end-point measurements of immune function [3], determination of activity of the hypothalamus–pituitary–adrenal axis [20], and detoxification [19], respectively. The effects of cortisol preincubation on the responsiveness of these genes to PCB exposure was also investigated to assess the interaction of stress and immune function under conditions representing a (prolonged) stress paradigm.

MATERIALS AND METHODS

Animals and reagents

Immature female rainbow trout, Oncorhynchus mykiss, (300–500 g) were maintained in 500-L-capacity aerated fiberglass tanks supplied with recirculating dechlorinated water containing 100 units/ml penicillin and 100 µg/ml streptomycin in order to remove any extraneous blood or blood clots. A mixed population of leucocytes and interrenal cells was obtained under sterile conditions by pushing the tissue through 100-µm nylon mesh using fresh L-15 medium and a syringe barrel. After three washes in L-15 medium, the cells were resuspended in medium containing antibiotics. Serum supplementation and further cell purification procedures were avoided to keep background levels of IL-1β expression down to a minimum [3]. After quantification, cells were seeded to a density of 0.5 × 10^6 cells/ml culture medium and a total volume of 2 ml/well added to six-well plates (Nunc, Rochester, NY, USA).

Cultivation of trout anterior kidney cells and lipopolysaccharide (LPS) responsiveness

Cell monolayers were incubated for up to 48 h at 22°C. Thereafter, cells were stimulated for a further 4 h at 22°C with Escherichia coli O127: B8 LPS at a concentration of 5 µg/ml by adding the appropriate volume to the culture dishes. At the end of the incubation period, cell culture dishes were decanted, and the culture medium was retained. Adherent cells were harvested into 1 ml of RNA-stat reagent (AMS-Biotech, Abingdon, UK) per sample, while nonadherent cells were pelleted by centrifugation and combined with the appropriate RNA-stat sample following the removal of the culture supernatant.

All samples were stored at −80°C before extraction of total ribonucleic acid (RNA).

Incubation of trout anterior kidney cells with PCB126: Time and dose relationship

Cell monolayers were incubated for up to 24 h at 22°C in the presence of PCB126 ranging in 1:100 dilutions from 1 µM to 0.001 nM. Cells were then harvested as described previously.

Preincubation and recovery from previous PCB126 exposure of trout anterior kidney cells: Effect on LPS responsiveness

Cell monolayers were incubated with 1 µM PCB126 for 2 and 24 h. Then, either the cells were stimulated with LPS as described previously or the PCB containing medium was removed and cells were incubated with fresh, naive medium for a further 24 h and then stimulated with LPS. At the end of the challenge period, cells were harvested as described previously.

Preincubation of trout anterior kidney cells with cortisol: Effect on PCB126 responsiveness

Cell monolayers were incubated with cortisol (320 ng/ml) for 1, 4, 12, and 24 h before a maximal 24-h incubation with 1 µM PCB126. At the end of the cortisol preincubation time, the medium was removed and quickly but carefully substituted by medium containing PCB126. The cells were then harvested at appropriate times as described previously.

RNA extraction, reverse transcriptase reactions, and real-time quantitative polymerase chain reaction (qPCR) analyses

Total RNA was isolated from the samples by using RNA-stat according to the manufacturer’s instructions. Five micrograms of total RNA were reverse transcribed using bioscript Moloney murine leukemia virus reverse transcriptase (Bioline, London, UK). The resulting complementary deoxyribonucleic acid (cDNA) was stored at −20°C before real-time qPCR analysis.

The following primers were used to detect expression levels of the following genes by real-time qPCR using a Light Cycler 1.2 (Roche, Penzberg, Germany): β-actin forward: 5’-TGCC AGATCTTCTCCATG-3’; reverse: 5’-ATGGAGGATGAAA TCGCC-3’; IL-1β forward: 5’-AGGGAGGCAGCTAC CACA-3’; reverse: 5’-GGGGCTGCCTTCTGACACAT-3’; GR forward: 5’-GATCCAGGTGACTGAAAC-3’; reverse: 5’-GCAGATGTTCCATTTGTAGT-3’; CYP 1A forward: 5’-GTTTTGACACCATTACACAGC-3’; reverse: 5’-AGGAAACGCTAGGTTGAA-3’. All qPCR products were initially verified by sequence analysis of the respective products, and initially real-time qPCR products were analyzed by agarose gel examination to control for the correct length of the products, and finally real-time qPCR products were analyzed by bioanalyzer gel examination to control for the correct length of the products.
as follows: initial denaturation: 7 min at 95°C, followed by 45 cycles of 95°C for 10 s, 59°C for 10 s, and 72°C for 15 s, followed by melting curve analysis. For calibration of the real-time qPCR data, serial dilutions of a column-purified (PCR Cleanup, Qiagen, Hilden, Germany) 250-bp β-actin PCR product were prepared. The DNA amount of the starting material was measured spectrophotometrically, based on absorbance values at 260 nm, with 1 A260 unit equivalent to 50 μg of DNA. Based on its molecular weight of 165,000 D, the amount of 2-ng PCR product (per 1 μl) is equivalent to $12 \times 10^{-15}$ moles (or 12 femoles) or $7.2 \times 10^6$ copies (1 mole = $6 \times 10^{23}$ copies). Further dilutions were performed with 10 mM Tris buffer, containing 10 ng/μl of inert plasmid DNA. Using this procedure, a further 1,000-fold dilution means $7.2 \times 10^6$ copies, with another 1,000-fold dilution giving 7,200 copies/μl. Using this dilution series in qPCR, number of cycles at threshold value (Ct values) for the corresponding β-actin copy numbers could be determined. Determination of Ct values for known amounts of input sample RNAs permits a direct determination of β-actin copy number per μg sample RNA. To validate extended comparisons for qPCR products from other genes, qPCR efficiencies were determined for β-actin (2.00) and the target genes IL-1β (1.98), GR (1.98), and CYP1A (1.99) (data not shown). Based on these very similar qPCR efficiencies, Ct values obtained for the target genes could be calculated as copies per μg RNA, based on the calibration curve obtained for β-actin. Furthermore, slight variations in the Ct values (±1) and the calculated β-actin copy numbers (up to fourfold) per μg RNA were corrected, assuming the same β-actin copy numbers per μg of RNA for all RNA samples. This permits an analogous correction for the copy numbers of all target genes.

**Statistical analysis**

Data represent means and standard errors of three independently performed cell isolation procedures for each experimental question. Data were analyzed performing one-way analysis of variance, followed by Tamhane’s posttesting, not assuming equal variances; $p < 0.05$ was accepted for statistical significance.

**RESULTS**

**Effect of time in culture**

Culturing anterior kidney cells for up to 48 h did not alter the LPS responsiveness of these cells with regard to the expression levels of IL-1β, GR, and CYP1A (data not shown).

**PCB exposure**

As shown in Figure 1A, all PCB doses (0.001 nM–1 μM) resulted in increased cytochrome P4501A gene expression levels. While doses of 0.001 and 0.1 nM induced maximal gene expression after 6 h of PCB incubation, higher doses (0.01 and 1 μM) did not reach a plateau of gene expression until after 12 h, with the plateau levels reached being threefold higher than the levels reached with the lower doses.

Figure 1B shows that all PCB doses applied resulted in a similar effect on expression levels of the GR gene. However, all PCB doses applied resulted in maximal expression levels of GR after 6 h of PCB exposure. Maximal expression levels reached after incubation with the highest PCB dose were threefold higher than levels reached after incubation with 0.001 and 0.1 nM PCB126 and twofold higher than levels reached after incubation with 0.01 μM PCB126.

A completely different picture was obtained when the expression of IL-1β was studied (Fig. 1C). First of all, 0.001 nM PCB had no effect on IL-1β expression. Using 0.1 nM of PCB126, an expression peak was reached after 4 h of PCB incubation ($p < 0.05$), followed by a decrease to levels observed in control cells not incubated with PCB126. Doses of 0.01 and 1 μM of PCB126 resulted in a sharp expression peak after 2 h of incubation, which was about threefold higher than the maximal expression levels obtained with the lower dose. This peak was followed by a steep decline, after 4 h, to IL-1β expression levels observed in control cells.

To further investigate the effect of PCB exposure on the ability of the anterior kidney cells to evoke a proinflammatory response, cells were incubated with PCB126 for 2 or 24 h. Incubating the cells with PCB126 for only 2 h (Fig. 2A) resulted in a significant increase in IL-1β gene expression levels. Removal of the PCB-containing medium after 2 h of incubation, followed by 24 h incubation in PCB-free medium, resulted in IL-1β gene expression levels similar to levels obtained in cells incubated without PCB. Stimulation with LPS
Cortisol incubation alone resulted in a significant increase ($p < 0.01$ for 1 and 4 h of cortisol preincubation) in CYP1A expression levels. After 12 and 24 h of cortisol preincubation, no PCB126-induced increase in CYP1A could be detected, and cortisol incubation alone also did not affect CYP1A expression levels.

Cortisol incubation for 4, 12, and 24 h resulted in a significant increase ($p < 0.05$ for 4 h and $p < 0.01$ for 12 and 24 h) in GR expression levels (Fig. 4). After 12 and 24 h of cortisol preincubation, no further increase in GR expression levels due to the PCB treatment was apparent. Incubation with PCB126 resulted in only those groups preincubated with cortisol for 1 and 4 h having PCB-induced increases in GR expression levels, similar to that seen in cells not preincubated with cortisol (see also Fig. 1B). In these groups, significant PCB-induced increases were determined after 2 h of PCB incubation ($p < 0.05$), increasing further over time and reaching maximal expression levels approximately threefold higher than in cells not preincubated with cortisol (Fig. 1B). However, no significant differences between any of the preincubation groups were observed after 24 h of PCB incubation.

Figure 5 shows the effect of cortisol preincubation on IL-1β expression. The sharp increase immediately after onset of the PCB126 exposure seen in cells not preincubated with cortisol did not occur. Instead, in samples given 1 and 4 h of cortisol preincubation, a time-dependent increase in IL-1β gene expression was observed, which became significant after 2 h of PCB incubation ($p < 0.05$). Maximal IL-1β expression levels were significantly lower in cells given 4 h of preincubation ($p < 0.05$) relative to those with 1 h of cortisol preincubation. Cortisol incubation alone resulted in a significant decrease in IL-1β expression after 4, 12, and 24 h. After 12 and 24 h of cortisol preincubation, PCB126 did not, at any time point, result in an increase in IL-1β gene expression level.

**DISCUSSION**

In this study, the effects of PCB126 incubation on gene expression levels of genes involved in detoxification as well as stress and immune function have been studied. Primary cell cultures of the anterior kidney or the head kidney only are frequently used as models to study various physiological parameters, such as immune responsiveness to LPS [3], EROD activities [14], or activity of the HPI axis [13]. Anterior kidney cell cultures are comprised of a variety of cell types, including leukocytes (macrophages, neutrophils, lymphocytes), constituting about 90% of the cells, and interrenal cells, catecholamine-producing cells, erythrocytes, and renal cells, making up the remaining 10% (J.W. Holland et al., University of Aberdeen, Aberdeen, Scotland, personal communication). The advantage of using such a heterogeneous cell population lies in its relatively close resemblance to the in vivo situation. The limitations of this model system are the obvious lack of the whole-body interplay of various organs and hormones produced and released in different parts of the body. However, a complex analysis of time- and dose-dependent effects of PCB exposure and cortisol preincubation would not have been possible in an in vivo approach, and therefore cell culture systems are a justifiable alternative. Cortisol levels in these cell suspensions are not detectable (J.W. Holland et al., personal communication); however, the physiological integrity of interrenal cells in vitro experiments has been demonstrated elsewhere [13]. It was shown in the study presented that incubating primary cultures of rainbow trout anterior kidney cells with
PCB affects gene expression in trout kidney

**Environ. Toxicol. Chem.** 24, 2005 3057

Fig. 3. Effect of cortisol preincubation on polychlorinated biphenyl 126 (PCB126)–induced cytochrome P4501A (CYP1A) gene expression levels. The effects of different cortisol preincubation times before PCB126 exposure on CYP1A gene expression levels are shown. All points represent means and standard errors of three independent cell preparations. Significant differences \((p < 0.05)\) to cells not incubated with PCB126 (black square on the insert, located approximately at the intersection of 0.0 on the x-axis and just below 5 on the y-axis) are indicated by asterisks. Open symbols indicate cells preincubated with cortisol only, and significant differences due to cortisol preincubation are indicated by dollar signs. Plus signs indicate significant differences \((p < 0.05)\) due to PCB exposure after cortisol preincubation. The insert depicts the first 1 h of PCB exposure in more detail, and significant differences observed in this time period are only shown in the insert.

Fig. 4. Effect of cortisol preincubation on polychlorinated biphenyl 126 (PCB126)–induced glucocorticoid receptor (GR) gene expression levels. The effects of different cortisol preincubation times before exposure on gene expression levels are shown. All points represent means and standard errors of three independent cell preparations. Significant differences \((p < 0.05)\) to cells not incubated with PCB126 (black square) are indicated by asterisks. Open symbols indicate cells preincubated with cortisol only, and significant differences due to cortisol preincubation are indicated by dollar signs. Plus signs indicate significant differences \((p < 0.05)\) due to PCB exposure after cortisol preincubation. The insert depicts the first 1 h of PCB exposure in more detail, and significant differences observed in this time period are only shown in the insert.

PCB126 affected gene expression levels of IL-1\(\beta\), CYP1A, and the glucocorticoid receptor.

**Effect of various doses of PCB126 on gene expression levels**

The highest PCB126 doses used in this study were in the same order of magnitude as PCB126 levels found in head kidney of tilapia after 5 d of oral exposure to PCB126 [17]. Despite reducing the highest dose (1 \(\mu\)M) in steps of 1:100 dilutions, only for IL-1\(\beta\) was the effect completely diluted out. Thus, incubating cells with 1 pM of PCB126 had no effect on IL-1\(\beta\) expression. Although the immunotoxic effect of PCBs has been demonstrated previously in mammals [12] and in teleost fish [21], this is, to the best of our knowledge, the first study to establish a subthreshold level of PCB126 exposure. Similarly, only sparse attention has been given to expression levels of other immune genes and, in particular, cytokines. Interleukin-1\(\beta\) in human mast cells was increased for up to 12 h of incubation with PCB153, with an increase observed already after 2 h (the first time point in this study) [22].
sharp increase of IL-1β expression levels seen in the present study, after only 30 min of PCB126 incubation, followed by an equally sharp decline after 2 h of incubation, indicates a strong but short-lived immunotoxic effect of PCB126 when used at doses of 1 and 0.01 μM. In a different study, an inhibition of the innate immune system in channel catfish (*Ictalurus punctatus*) after exposure to PCB was observed [23]. However, in this study [23] the fish were injected and sampled 14 and 21 d after injection; hence, a stimulation of the innate immune system at an earlier time point might have been missed.

A possible explanation for the observed increase in IL-1β gene expression levels could be the fact that coplanar PCBs like PCB126 can induce nuclear factor-κB (NF-κB) [24] and mitogen-activated protein kinase (MAPK) [25] expression, which are known to increase IL-1β expression levels [25,26]. The relatively short and transitory effect of PCB126 on IL-1β expression, on the other hand, might be explained by the fact that GR gene expression levels were high enough to exhibit their known antagonist effect on NF-κB and MAPK levels [27] only after more than 2 h of PCB exposure, hence leading to the observed decrease in IL-1β levels.

The effects of PCBs and related environmental pollutants on the stress response in fish have been studied to some extent. Stress hormone levels in exposed fish have been examined, and the responsiveness of exposed fish to additional stressors, like net confinement [17] or in vivo [28] or in vitro adrenocorticoid hormone challenge [17], have been determined. Such studies revealed an impaired responsiveness of the interrenal tissue to elicit a stress response. Furthermore, comparing plasma cortisol levels of unstressed fish either exposed to PCB in a laboratory study [17] or caught from polluted environments [10] did not show increased plasma cortisol levels, indicating that the toxicants themselves did not evoke a stress response in these fish. However, the present data on PCB126-induced glucocorticoid receptor (GR) expression levels may suggest a different explanation, namely, that substances like PCB126 do indeed evoke a stress response, resulting in increased plasma cortisol levels, which in a feedback mechanism stimulates the increase in GR production, possibly resulting in the binding of free cortisol and its excretion. Alternatively, the PCB incubation might result directly in increased GR levels, which would immediately bind any (newly) synthesized cortisol, and such an increase could be explained by the structural similarities between PCBs and cortisol [29].

The effects of various environmental pollutants, including PCBs and PCB126, on cytochrome P4501A, measured either as EROD activities and/or CYP1A protein or as messenger (m)RNA expression, has been documented in various fish organs, like liver, gill, intestine, head kidney, spleen, and brain [14,30–32]. In line with these studies, we observed an increase in CYP1A gene expression levels with increasing PCB126 incubation time and dose, indicating the activation of detoxifying activity in the primary cell culture system used here.

**Effects of PCB exposure on LPS-induced IL-1β gene expression**

To further investigate the effect of PCB exposure on the ability of leukocytes to evoke a proinflammatory response, cells were stimulated with LPS after the PCB126 exposure. Lipopolysaccharide is known to stimulate the innate immune response not only in mammals [33] but also in fish [3]. Thus, IL-1β gene expression in rainbow trout has been shown to be induced by bacterial LPS [3]. The results obtained in the present study indicate that LPS-induced IL-1β gene expression is not impaired by PCB incubation, suggesting that LPS stimulates IL-1β by a different pathway. This needs to be investigated further, but it appears unlikely that the nuclear transcription factor NF-κB is a candidate involved in such a hypothetical alternative stimulatory pathway since it has been shown that PCB153 rapidly transforms NF-κB from its inactive to its active form [22], as it occurs with LPS [34]. The data
obtained in the present study are in contrast to data obtained in murine splenocytes, which showed depressed LPS-induced proliferation rates after incubation with Aroclor mixtures [35], but this might be explained by a different sensitivity of different organs to PCBs or by the fact that mixtures of chemicals often exhibit different responses than do single compounds. However, the data obtained here show that the decline in IL-1β expression levels after more than 4 h of PCB incubation is not due to permanent damage or exhaustion inflicted on the anterior kidney leukocytes. Tissue or cell exhaustion, described as morphological alteration subsequently resulting in cellular dysfunction (e.g., inability regarding hormone production and/or release), has been suggested to be the cause for impaired stress responsiveness after toxicant exposure [10]. Furthermore, these results support the previous notion that PCB exposure in itself is not stressful for teleost fish, such as rainbow trout and tilapia [31], because it has been reported that exposure to LPS at the same time as, or immediately following, a stressor resulted in down-regulation of proinflammatory cytokines, including IL-1β [36].

**Effect of cortisol preincubation on PCB126-induced gene expression levels**

In agreement with the findings in the present studies, it has been reported previously that cortisol incubation results in decreased IL-1β expression in vitro and in vivo [3,4]. However, the present studies are in contrast to studies on lake trout (*Salvelinus namaycush*) thymocytes [37]. Incubating lake trout thymocytes with cortisol increased the toxicity of Aroclor to these cells. These differences might be accounted for by a different susceptibility of these different cell populations. However, the effect on the time dependency of PCB126-induced IL-1β expression, and in particular the abolished responsiveness to PCB126 of the anterior kidney cells to express IL-1β after 12 and 24 h of cortisol preincubation, may indicate a cortisol-induced dysfunction of the kidney leukocytes. Such a dysfunction could involve the inability of the leukocytes to elicit an inflammatory response, resulting in increased IL-1β expression levels.

Previous studies investigating the effect of stress on GR expression in fish leukocytes revealed increased GR levels in coho salmon (*Oncorhynchus kisutch*) leukocytes after acute stress and after cortisol incubation [38]. Furthermore, it could be shown that stress and cortisol inhibit LPS-induced B-cell proliferation and antibody production in vitro and in vivo, and it has been suggested this is due to cortisol affecting the immune system via receptor-mediated suppression of cytokines [39]. Glucocorticoid receptor gene expression levels were increased after all cortisol preincubination regimes used in the study presented, and this is in agreement with the studies by Sathiyaa and Yijayan [40]. After 1 and 4 h of cortisol preincubation, the anterior kidney cells showed a similar response to PCB incubation as cells not exposed to cortisol, but maximal levels were approximately fourfold higher after cortisol incubation, indicating a sensitization of the interrenal cells. Overall, this indicates that under an acute stress regime, fish might still exhibit a PCB-induced GR response similar to unstressed fish. However, after 12 and 24 h of cortisol preincubation, GR levels were about 15-fold above levels obtained in control cells not incubated with cortisol, but no further increase due to PCB incubation could be detected. This might indicate either an already maximal stimulation of GR expression after the cortisol incubation or that under a prolonged stress regime the amount of GR molecules present are not capable of binding any further produced free cortisol, if indeed PCB exposure does result in increased cortisol levels. However, since the (cortisol-mediated) regulation of GR expression is rather complex and increased GR mRNA levels are, at least in trout hepatocytes, not strictly correlated with GR protein levels [40], any effects of PCB treatment on receptor recycling mechanisms cannot be ruled out.

Unlike the previously mentioned parameters investigated in this study, the effects of cortisol on cytochrome P450 or at least EROD activities have been studied to some extent, even though most studies have focused on induction patterns in liver or hepatocytes. It could be shown that cortisol increased the effects of β-naphthoflavone on EROD activities in trout hepatocytes [15]. Similarly, it was reported that confinement stress mediated EROD activities in head kidney of tilapia fed a PCB-containing diet [16]. Thus, the increase of CYP1A gene expression after 1 and 4 h of cortisol incubation is in line with these studies. It has been suggested that cortisol mediates EROD activities and possibly CYP1A gene expression via glucocorticoid receptors [41], which would be supported by the findings in the present study, where GR and CYP1A gene expression levels are increased after 1 and 4 h of cortisol preincubation and increase further over the time course of PCB exposure. The abolished CYP1A response 12 and 24 h after cortisol incubation, together with no increase due to the PCB exposure, is in contrast to this suggested mode of action and questions the effectiveness of CYP1A under chronic stress regimes. However, the apparent controversy over the lack of increased CYP1A expression after 12 and 24 h of cortisol preincubation and the large increase of GR expression could be due either to expression of different isoforms of the receptor with different physiological properties or to a biphasic response of CYP1A expression to increasing cortisol preincubation time, as has been observed when toxicant doses are increased [42].

**CONCLUSION**

It is concluded that primary cultures of rainbow trout anterior kidney cells represent a good model system to study the effects of toxicants like PCB126 on genes involved in stress and immune responses since the data obtained in the present study are basically corroborated by data obtained from in vivo studies. The results presented here further emphasize the necessity to control, or at least account for, additional environmental parameters like stress status of the animals when sampling fish from the wild or even under laboratory conditions. Thus, prolonged incubation with cortisol, which could represent a chronic stress paradigm, is shown here to affect not only GR expression levels but also expression levels of cytochrome P450 and IL-1β, both frequently used as biomarkers, the latter to examine the immune status of fish.

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