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PCB-Induced Hepatic CYP1A Induction is Associated with Innate Immune Dysfunction in a Feral Teleost Fish

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Polychlorinated biphenyls (PCBs) are well known for the plethora of toxic effects they can induce. The immunotoxic potential of these compounds is well documented in mammals and evidence is rapidly accumulating demonstrating similar effects in fish. For example, a single intraperitoneal (IP) exposure of juvenile Chinook salmon to Aroclor 1254 (54 mg/kg) suppressed antibody forming cell (AFC) numbers (compared to controls) in fish examined 10 wk post-PCB injection (Arkoosh et al. 1994). Humoral immunity was also suppressed in Japanese medaka following IP exposure to PCB 126 (1.0 μg/g body weight [BW]); immunosuppression appeared 3 d post-treatment and persisted for up to 14 d (Duffy et al. 2002). In channel catfish, plasma antibody responses were significantly depressed (compared to controls) at 21 d following IP exposure to 1.0 μg PCB 126/g BW (Regala et al. 2001). PCB exposure has also been shown to affect nonspecific immunity of fish including phagocytosis and the production of reactive oxygen intermediates (ROI) such as superoxide anion ($O_2^{\cdot-}$); as in mammals, fish phagocytes upon stimulation produce ROIs that participate in a variety of functions including antimicrobial defense. For example, oxidative burst activity by kidney phagocytes was suppressed in channel catfish 3 d following a single IP injection of 1.0 μg PCB 126/g BW; effects of PCBs on ROI persisted for up to 14 d (Rice and Schlenk 1995; Regala et al. 2001). In the same study, exposure of catfish to a one hundred-fold lower PCB concentration (i.e., 0.01 μg/g BW) significantly reduced oxidative burst activity (compared to controls), but only after 1 and 2 wk (Regala et al. 2001). Dose and time-dependent alterations in phagocyte-mediated $O_2^{\cdot-}$ production by PCB 126-exposed Japanese medaka have also been reported (Duffy et al. 2003). In this study, unstimulated $O_2^{\cdot-}$ production by kidney phagocytes from juvenile and adult fish treated with 1.0 μg PCB 126/g BW was significantly increased (compared to controls) 14 d post-exposure; PCB exposure had no effect on unstimulated $O_2^{\cdot-}$ production 3 d following treatment (Duffy et al. 2003). In addition, feral fish populations exposed to PCB-contaminated sediments have demonstrated altered phagocytic activity (Lacroix et al. 2001). Phagocytosis by macrophages recovered from American plaice exposed to either PCB contaminated sediment (1500 ng total PCB/g dry weight [DW]) from Baie des Anglais, Quebec, or to beach sand (13.6 ng total PCB/g DW) was measured after 1, 2 and 3 mo of exposure. Kidney macrophages recovered from fish exposed to contaminated sediment had reduced

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phagocytic activity (compared to controls) at all post-exposure time points examined (Lacroix et al. 2001).

Despite an abundance of studies demonstrating the immunotoxic potential of PCBs, a clear mechanism of toxicity has yet to be determined. Generally, activation of the aryl hydrocarbon receptor (AhR), specifically by coplanar PCB congeners, is thought to play a role in mediating PCB-induced immunotoxicity in mammals (Silkworth and Grabstein 1982). Given that fish can be chronically exposed to PCBs in their natural environment and are sensitive to PCB-induced immunomodulation and AhR activation (Rice and Schlenk 1995), a better understanding of the potential mechanism of PCB-induced immunotoxicity in fish is warranted. Thus, the current study sought to determine what association, if any, exists between AhR activation (i.e., CYP1A induction) and immunotoxicity in a feral fish species. In addition, results from this study will provide a better understanding of the validity of the fish model for species extrapolation and comparative immunotoxicity studies.

MATERIALS AND METHODS

Killifish were collected from Long Island Sound (Milton Harbor, Rye, NY), acclimated to room temperature (25°C), and depurated for at least one month prior to use. Fish, maintained on a 16/8 h light/dark cycle in artificial seawater (salinity 18-22 ppt) prepared using declorinated tap water, were fed daily (except on the day of PCB exposure) a diet of Tetramin® Richmix flake food. The PCB congener, purchased from AccuStandard (New Haven, CT), had a purity value of 100%. On the day of PCB exposure, each fish (1 – 2 g) received a single IP injection of either corn oil (vehicle control) or PCB 126 at a concentration of 0.01 or 1.0 μg/g BW. Selection of the PCB doses were based upon previously published studies that demonstrated PCB-induced immunomodulation in other fish species at these same levels (Rice and Schlenk 1995; Duffy et al. 2003). Each treatment group consisted of 3 – 5 fish per 40L tank to avoid any crowding effect. Intra- and extracellular $O_2^{\cdot-}$ production by kidney phagocytes was examined as a measure of innate immune function at 1 and 7 d post-PCB injection. As an indicator of AhR activation, hepatic CYP1A levels were measured at the same time points post-injection as the immune function parameters (1 and 7 d), as well as two additional post-injection time points (3 and 14 d) to determine CYP1A induction kinetics. Briefly, at the appropriate post-exposure time point, fish were sacrificed, kidneys aseptically removed and placed in fish physiological saline (FPS) (Zelikoff et al. 1996) supplemented with 1% (w/v) glucose. Kidneys, pooled within a given exposure group (3 –5 fish) so as to ensure the appropriate number of cells for the experiment, were homogenized, centrifuged and resuspended in supplemented FPS. Cell viability and total kidney cell numbers were determined by trypan blue exclusion and hemocytometer counting, respectively (Zelikoff et al. 1996). Intra- and extracellular $O_2^{\cdot-}$ production were measured using nitroblue tetrazolium (NBT) reduction and fericytochrome c reduction, respectively as described previously by Zelikoff et al. (1996). Briefly, phorbol 12-myristate 13-acetate (PMA)–stimulated $O_2^{\cdot-}$

production by kidney phagocytes was measured by the $O_2^{\cdot-}$ - induced reduction of NBT (intracellular) or fericyctochrome c (extracellular) in the presence of superoxide dismutase (SOD). For intracellular $O_2^{\cdot-}$ production, absorbance was determined spectrophotometrically after a 30 min incubation (at 30°C) at 630 nm. For extracellular $O_2^{\cdot-}$ production, absorbance was measured at 550 nm after incubation (at 30°C) for either 0, 10, 20, 30, 60, 90 or 120 min. Data is presented as mean (n = 3 – 5 replicates) stimulation index (PMA-stimulated $O_2^{\cdot-}$ production / unstimulated $O_2^{\cdot-}$ production). Individual livers from sacrificed fish were flash frozen in liquid nitrogen and stored at -70°C until used to measure CYP1A protein levels by ELISA as described by Carlson et al. (2002). Briefly, protein levels were measured by ELISA using a monoclonal anti-trout antibody (C10-7) as the primary antibody and an anti-mouse IgG alkaline phosphatase conjugate as the secondary antibody. Data for each treatment group was presented as mean absorbance of the reduced substrate (*p*-nitrophenyl phosphate) at 405 nm. Data were analyzed by one-way analysis of variance (ANOVA), followed by Fisher's post-hoc testing when necessary. Significant differences between control and experimental groups were accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Exposure of killifish to PCB 126 doses ranging from 0.01 – 1.0 μg/g BW had no effect on host survival (> 98%) for up to 14 d post-injection, and no differences in kidney cell numbers (i.e., 1.5 x 10^6 cells/fish) or viability (> 95%) were observed between control and any PCB-treatment group. While extracellular $O_2^{\cdot-}$ production was unaffected by PCB exposure, intracellular $O_2^{\cdot-}$ production was depressed 7 d post-injection in killifish treated with the highest PCB dose (1.0 μg/g BW); no effects on intracellular $O_2^{\cdot-}$ production were observed 1 d post-injection (Figure 1).

Measurement of hepatic CYP1A levels at 1, 3, 7 and 14 d post-injection revealed a time-dependent effect of PCB 126 on protein induction (Figure 2). While after 1 d CYP1A protein was not induced in any PCB treatment group, significant induction became apparent at 3 d post-injection in fish treated with the highest PCB 126 dose; protein levels in the fish remained elevated for up to 14 d post-injection. Killifish treated with the lowest dose of PCB 126 showed no hepatic CYP1A induction at any post-exposure time point.

Because intracellular $O_2^{\cdot-}$ production was significantly altered 7 d, but not 1 d post-injection, PCB-induced immunotoxicity may be delayed in its onset. Similar time-dependent effects of PCBs on intracellular $O_2^{\cdot-}$ production have been observed in other immunotoxicity studies utilizing different fish species (Rice and Schlenk 1995; Duffy et al. 2002; Duffy et al. 2003). Similarly, PCB-induced CYP1A protein induction has also been shown to be time-dependent in a variety of fish models (Rice and Schlenk 1995; Schlezinger and Stegeman 2001; Duffy et al. 2003). In the current study, PCB 126 only induced CYP1A protein at the highest PCB dose (i.e., 1.0 μg/g BW) and only at 3, 7 and 14 d post-injection. Given that intracellular $O_2^{\cdot-}$ production was only suppressed at a PCB dose and

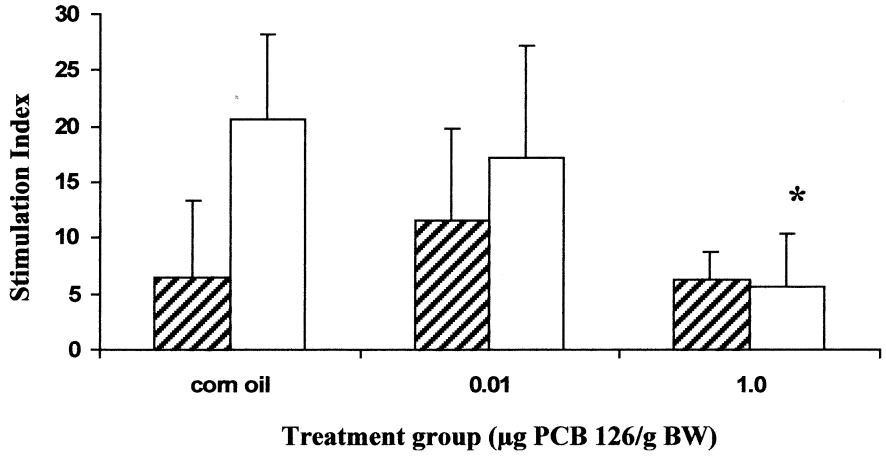

Figure 1. Intracellular $O_2^{\cdot-}$ production 1 and 7 d post-PCB injection. Data is presented as stimulation index (PMA-stimulated $O_2^{\cdot-}$ production / unstimulated $O_2^{\cdot-}$ production). Mean (n = 3 – 5 experiments) ± SD. *Significantly different from time-matched vehicle control ($p < 0.05$).

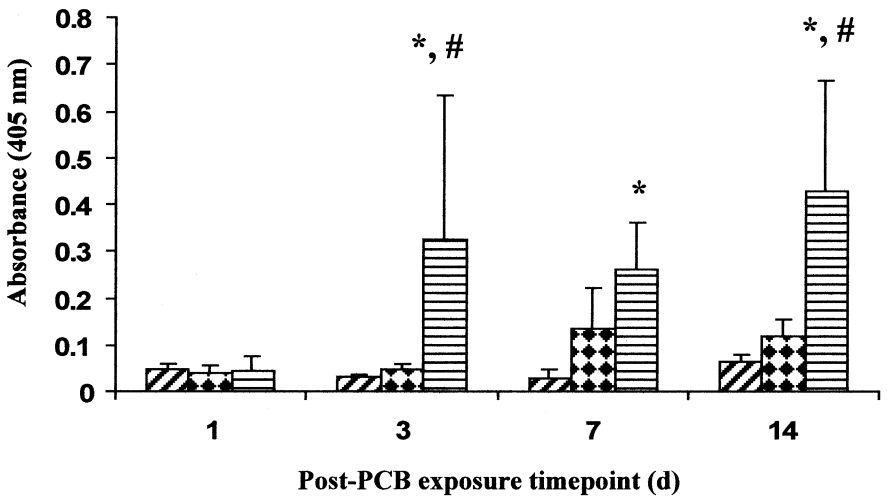

Figure 2. Killifish were IP injected with either corn oil 0.01 or 1.0 µg PCB 126/g BW and hepatic CYP1A protein levels measured 1, 3, 7 and 14 d later. Data for each treatment group is presented as mean absorbance of the reduced substrate at 405 nm. Mean (n = 3 – 6 experiments) ± SD. *Significantly different from time-matched vehicle control ($p < 0.05$). #Significantly different from 0.01 µg PCB/g BW treatment group ($p < 0.05$).

post-exposure time point that induced CYP1A (7 d), an association between CYP1A induction and immunosuppression can be inferred.

One possible mechanism of PCB-induced immunotoxicity is thought to involve activation of the AhR. Several investigations using Ah locus positive (Ah+) or negative (Ah-) mice have demonstrated the importance of this pathway in PCB-induced humoral immune suppression (Silkworth and Grabstein 1982; Silkworth et al. 1984; Kerkvliet et al. 1990). In one study, Ah+ mice injected with 3,3',4,4'-tetrachlorobiphenyl, an AhR agonist, demonstrated a suppressed AFC response to injected sheep red blood cells (SRBCs); Ah- mice treated with the same PCB congener showed no response (Silkworth and Grabstein 1982). In addition, neither strain of mice demonstrated any suppression of the AFC response when treated with a PCB congener having low affinity for the AhR. In the same study, hepatic cytochrome P-450 levels were induced in the Ah+ strain of mice only, further establishing a correlation between PCB-induced AhR activation and immunotoxicity (Silkworth and Grabstein 1982). A similar relationship has also been determined for other PCB congeners (Silkworth et al. 1984) and immune responses (Kerkvliet et al. 1990). For example, while Ah+ mice treated with a PCB congener having a high affinity for the AhR demonstrated a suppressed cytotoxic T-lymphocyte (CTL) response upon challenge with syngeneic tumor cells, CTL activity remained unaltered in mice congenic at the Ah locus (Ah-) or in Ah+ mice exposed to a PCB congener with low AhR affinity (Kerkvliet et al. 1990).

PCBs have also been shown to cause a general state of oxidative stress in teleost fish (Schlezinger and Stegeman 2001). When coplanar PCBs such as PCB 126 bind the AhR, CYP1A typically becomes upregulated. However, some PCBs have been shown to uncouple the catalytic cycle of CYP1A, thus leading to the production of reactive oxygen species (ROS) and a decrease in CYP1A protein levels and enzymatic activity (Schlezinger and Stegeman 2001). Uncoupling of the catalytic cycle of CYP1A following exposure to PCB 126 has been demonstrated in scup (*Stenotomus chrysops*) (Schlezinger and Stegeman 2001). In this case, scup treated with 0.01 μg PCB 126/g demonstrated significant hepatic CYP1A induction beginning on day 7 post-exposure and persisting until day 18; exposure of scup to a 100-fold higher PCB 126 dose failed to induce hepatic CYP1A until 16 d post-exposure. Since significant CYP1A induction was observed in this study in exposed killifish as early as 3 d post-exposure, treatment with 1.0 μg PCB 126/g BW does not appear to uncouple the CYP1A catalytic cycle. Furthermore, given that PCB suppressed intracellular $O_2^{\cdot-}$ production at this same dose, oxidative stress does not appear to be a relevant issue. Contrasting results observed between the killifish and scup studies may be due to discrepancies in species sensitivity to PCB 126 exposure. Studies utilizing other fish species have demonstrated PCB-induced CYP1A induction patterns similar to those observed in the present study (Rice and Schlenk 1995; Duffy et al. 2003).

In summary, the current studies demonstrate the importance of post-exposure duration for observing PCB-induced immunotoxicity and suggest that PCB-

induced activation of the AhR plays an important role in the immunotoxicity of coplanar PCB congeners.

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