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Oxidative Stress Induced in PCB 126-Exposed Northern Leopard Frogs, *Rana pipiens*

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Northern leopard frogs *Rana pipiens* exposed to PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl) were examined for hepatic oxidative stress. In a dose-response study, northern leopard frogs were injected intraperitoneally with either PCB 126 in corn oil (0.2, 0.7, 2.3, or 7.8 mg/kg body weight) or corn oil alone. In a time-course study, frogs received 7.8 mg/kg or corn oil alone, and were examined at 1, 2, 3, and 4 wk after dosing. Hepatic concentrations of reduced glutathione (GSH), thiobarbituric acid-reactive substances (TBARS), and total sulfhydryls (total SH), as well as activities of glutathione peroxidase (GSH-P), GSSG reductase (GSSG-R), glucose-6-phosphate dehydrogenase (G-6-PDH), and glutathione *S*-transferase (GSH-S-T) were measured. In the dose-response experiment, few effects were apparent 1 wk after dosing. In the time-course experiment, significant changes were observed in the 7.8-mg/kg group at 2 wk or more posttreatment. Hepatic concentrations of GSH and TBARS were higher than in corresponding controls at wk 3 and 4; the activities of GSSG-R and GSH-S-T were higher than in controls at wk 2 and 4; and the activity of G-6-PDH was increased at wk 2 and 4. These data collectively indicate that altered glutathione metabolism and oxidative stress occurred and were indicative of both toxicity and induction of protective mechanisms in frogs exposed to PCB. A similar delay in response was reported in fish and may relate to lower metabolic rate and physiological reactions in ectothermic vertebrates.

Polychlorinated biphenyls (PCBs) are industrial environmental contaminants that are persistent, lipophilic, and ubiquitous throughout the global ecosystem in fish, birds, and mammals including humans (Safe, 1984; Eisler, 1986; Hoffman et al., 1996b; Giesy & Kannan, 1998; DeRosa et al., 1998; Rice et al., 2003). Adverse responses produced by exposure to PCBs in mammals include thymic atrophy, a wasting syndrome, immunotoxic effects, reproductive impairment, porphyria and related liver damage, and induction of specific isozymes of the cytochrome P-450 system (Kimbrough, 1980; Safe, 1984, 1990; Giesy and Kannan, 1998; McKinney & Waller, 1998; Rice et al., 2003).

In the past, studies reported on exposure of northern leopard frogs (*Rana pipiens*) to aryl hydrocarbon receptor agonists using direct chemistry, biochemical markers, and toxicokinetics (Huang & Karasov, 2000). The results indicated an almost complete absorption of ingested PCBs and a slow elimination rate ($t_{1/2}$ = 763 d) compared to hamster, rat, guinea pig, herring gull, rainbow trout, and monkey. The induction thresholds of activities for 4 cytochrome P-450-associated monooygenase detoxifying enzymes, ethoxyresorufin *O*-dealkylase (EROD), methoxy-ROD (MROD), benzyloxy-ROD (BROD), and pentoxy-ROD (PROD), ranged between 0.7 and 2.3 mg/kg PCB 126, while the increased enzyme activities persisted throughout the 4-wk experiment (Huang et al., 1998). In addition, PCB 126-treated frogs showed that cytochrome P-450 1A (CYP1A) was induced in hepatocytes, epithelium of nephronic duct, vascular endothelium of a variety of tissues, and dermal epithelial cells of mucous glands and serous glands (Huang et al., 2001).

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To our knowledge, there have been no studies on oxidative stress produced by PCB exposure in amphibian species. Increased hepatic oxidative stress was reported following exposure to polyhalogenated hydrocarbons in birds and mammals, including exposure to PCB 126 in American kestrels, *Falco sparverius* (Hoffman et al., 1996a; Fernie et al., 2000; Fairbrother et al., 2004), TCDD in Sprague-Dawley rats (Stohs et al., 1990), TCDD in C57Bl/6J mice (Hori et al., 1997), and PCBs in mink, *Mustela vison* (Hoffman et al., 1992). Glutathione *S*-transferases (GSH *S*-transferases) are a multigene superfamily of dimeric, multifunctional, and soluble enzymes that play an essential role in protecting organisms from oxidative damage to DNA and lipids. For instance, GSH *S*-transferases conjugate glutathione (GSH) with electrophilic centers in a variety of compounds (e.g., epoxides) in the first step of the mercapturic acid pathway. Under oxidative stress, the peroxidase activity of GSH peroxidase reduces lipid hydroperoxides by nucleophilic attack of GSH on the electrophilic oxygen. The oxidized GSH (GSSG) is subsequently recycled via a coupled reaction involving GSSG reductase and G-6-PDH.

In this study, hepatic oxidative stress following exposure of 3,3′,4,4′,5-pentachlorobiphenyl, PCB 126, was examined in northern leopard frogs. Enzymes related to GSH metabolism were measured, including glutathione peroxidase (GSH peroxidase), glutathione reductase (GSSG reductase), glucose-6-phosphate dehydrogenase (G-6-PDH), and glutathione *S*-transferase (GSH *S*-transferase), as well as hepatic concentrations of GSH, total sulfhydryls (total SH), and thiobarbituric acid reactive substances (TBARS) as a measure of lipid peroxidation.

MATERIALS AND METHODS

Chemical Source and Animal Care

3,3′,4,4′,5-Pentachlorobiphenyl (purity >99%) was purchased from Cambridge Isotope Laboratories (Woburn, MA). The purity was reanalyzed by the Department of Water Sciences at the University of Wisconsin at Madison, and was >99.5% of the total PCB quantity found in the stock solution. Enzyme substrates were purchased from Molecular Probes (Eugene, OR). All other chemicals were of the highest quality available from commercial sources.

Sixteen female and 50 male leopard frogs (25.61 ± 4.39 g; mean ± SD; $n = 66$) were collected from the Leopold Memorial Reserve, Baraboo, WI. Until the experiment, they were housed for 2 mo in 4 fish tanks with running dechlorinated water. The tanks were tilted so that frogs could swim in the water pool or sit on dry substrate. Frogs were fed crickets and mealworms ad libitum. The room temperature was set at 20°C under a 12-h L:12-h D photoperiod.

Two weeks before the experiment was conducted, frogs were housed individually in Rubbermaid tubs (58.4 × 42.5 × 22.9 cm) lined with high-density polyethylene plastic bags. The liners were changed every 6 d. Water was supplied in small plastic dishes and changed every other day. Each frog was fed 7 crickets every 3 d, which permitted maintenance of body weight or growth. Mealworms were also provided intermittently starting at wk 2. Vitamins and minerals were provided every 6 d by dusting them onto crickets. In both holding regimes, frogs ate and responded to humans normally, and no skin diseases were noted.

Frogs were fasted 24 h before a single intraperitoneal injection of chemical solution. Each injection was 2 ml/kg and contained either a solution of PCB 126 in corn oil at a designated dosage or corn oil alone.

Dose-Response Experiment

The dosage levels were 0, 0.2, 0.7, 2.3, or 7.8 mg/kg PCB, and frogs were sacrificed by decapitation 1 wk after dosing. Twenty-seven frogs were used, with five or six individuals randomly assigned to each treatment group. This included 18 males and 9 females. Body weights of the frogs prior to treatment did not differ significantly among groups. This study and the time-course study described next were not designed to examine for gender-related responses to PCB 126, since Huang et al. (1998) did not find any PCB 126 gender-related differences for either CYP1A enzyme levels or induction.

Time-Course Experiment

Frogs received 7.8 mg/kg PCB or corn oil alone, and were sacrificed at 1, 2, 3, or 4 wk after dosing. In total, 42 frogs (including the controls and 7.8 mg/kg from the already described dose-response experiment for the wk 1 data) were used with 4–6 individuals randomly assigned to each treatment group for wk 2, 3, and 4. This included 32 males and 10 females. Body weights of the frogs prior to treatment did not differ significantly among groups.

Measurements of Oxidative Stress-Related Parameters

These measurements were conducted with aliquots of the same livers and at approximately the same time that EROD and other cytochromes P-450 enzyme activities were determined. Portions of the liver were minced and homogenized (1:10 w/v) in ice-cold 1.15% KCl-0.01 *M* Na,K-phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 × g for 20 min at 4°C and the resultant supernatant was used for assays for enzymes related to glutathione metabolism and antioxidant activity. Liver glutathione peroxidase (GSH peroxidase, EC 1.11.1.9; coupled reaction at 30°C with glutathione reductase using cumene hydroperoxide) and glutathione reductase (GSSG reductase, EC 1.6.4.2) activities were recorded spectrophotometrically by micromethods using a centrifugal analyzer as described by Jaskot et al. (1983). Glutathione *S*-transferase (GSH *S*-transferase; EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene as the substrate (Habig et al.,

1974) and glucose-6-phosphate dehydrogenase (G-6- PDH; EC 1.1.1.49) activity according to the method of Lohr and Waller (1974) using the centrifugal analyzer. Reduced glutathione (GSH) as nonprotein sulfhydryl and total hepatic sulfhydryl concentrations (total SH) were measured according to Sedlak and Lindsay (1968). Protein-bound sulfhydryl (PBSH) concentrations were calculated as the difference between total SH and GSH concentrations. Thiobarbituric acid-reactive substances (TBARS) were measured as an estimate of hepatic lipid peroxidation using the method described by Aust (1985). Standard curves were generated for the assay using malondialdehyde tetraethyl acetal. Crude homogenate and 10,000 × g supernatant protein concentrations were determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical Analysis

In the dose-response study relating PCB 126 dose levels with parameters of oxidative stress, analysis of variance (ANOVA) was performed followed by Dunnett's tests for comparison between treatment groups and the control group. In the time-course study, two-tailed *t*-tests were used to compare the single treatment level (7.8 mg/kg) used with the corresponding control group for that time interval. When homogeneity of variance was lacking, log transformations of data were done. Statistical significance in all analyses was set at $\alpha = .05$.

RESULTS

Throughout the experiment, frogs responded normally to human disturbance and exhibited no anorexia, skin or organ lesions, and mortality. At the time of killing, all frogs had food residues in their stomachs, indicating they received enough food. Huang et al. (1998) previously reported that the pretreatment body weights of these leopard frogs did not differ significantly among treatment groups and dosage was not a significant factor in explaining variation in body weight or liver weight with time (Huang et al., 1998).

Dose-Response Experiment

In the dose-response experiment relating PCB 126 dose levels with the activities of GSH peroxidase, GSSG reductase, G-6-PDH, and GSH *S*-transferase, only GSH peroxidase at the 2.3 mg/kg dose group was significantly lower than the control (Figure 1, $n = 5$). The levels of GSH, total SH, and TBARS, an indicator of lipid peroxidation, were not significantly different from those of control groups (data not shown, $n = 5$–6).

Time-Course Experiment

In the time-course experiment relating subchronic exposure from a single ip injection of PCB 126, significant differences from the control groups were observed over time for activities of GSSG reductase, G-6-PDH, and GSH *S*-transferase, as well as for concentrations of GSH, total SH, and TBARS. The levels of GSH and TBARS in the single dosed group (7.8 mg/kg) were significantly higher than for controls at wk 3 and 4 (Figures 2 and 3, $n = 5$–6). The activity of GSSG reductase in the 7.8 mg/kg dosage group at wk 2 and 4 were significantly higher than controls (Figure 4, $n = 5$–6). The activity of GSH *S*-transferase in the 7.8 mg/kg dosage group was significantly higher than controls at wk 2 and 4 (Figure 5, $n = 5$–6). The activity of G-6-PDH in the 7.8 mg/kg dosage group at wk 2 was significantly higher than controls (Figure 6, $n = 5$–6), whereas at wk 4 the activity was marginally significantly higher than controls ($p = 0.0597$, $n = 5$–6). The concentrations of total SH at 7.8 mg/kg were significantly higher than controls at wk 3 (Figure 7, $n = 5$–6).

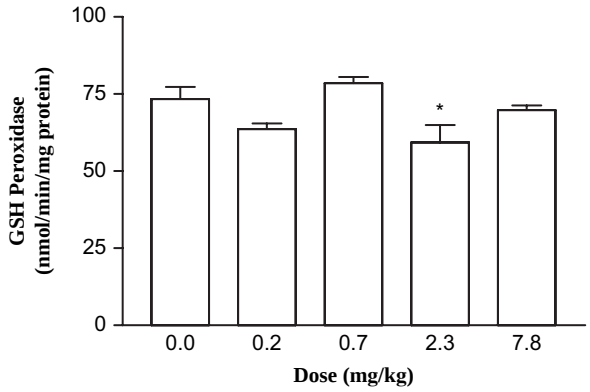

FIG. 1. Dose-response study of activity of hepatic GSH peroxidase. Asterisk indicates significantly different from controls ($p < .05$, $n = 5$).

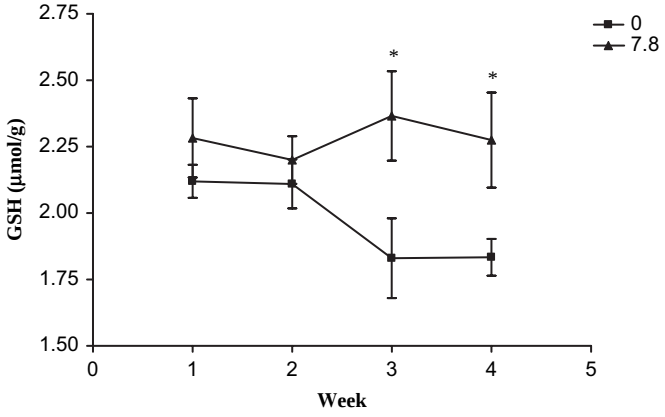

FIG. 2. Time-course study of concentrations of hepatic GSH. Asterisk indicates significantly different from controls ($p < .05$, $n = 4$–6). Doses were mg/kg.

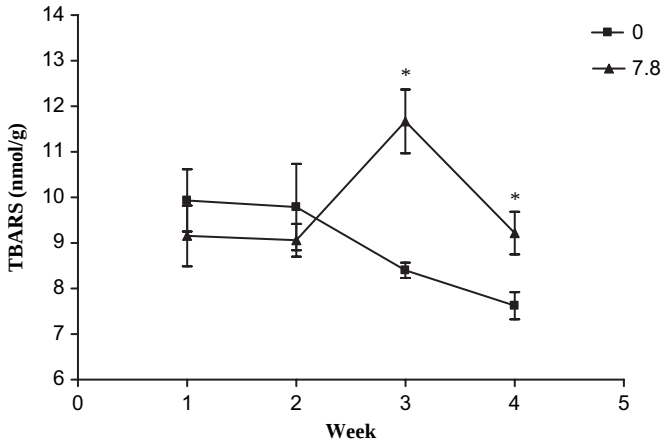

FIG. 3. Time-course study of concentrations of hepatic TBARS. Asterisk indicates significantly different from controls ($p < .05$, $n = 4–6$). Doses were mg/kg.

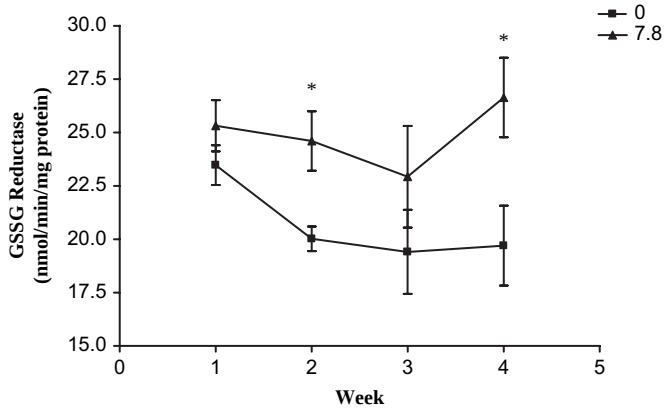

FIG. 4. Time-course study of activities of GSSG reductase. Asterisk indicates significantly different from controls ($p < .05$, $n = 4–6$). Doses were mg/kg.

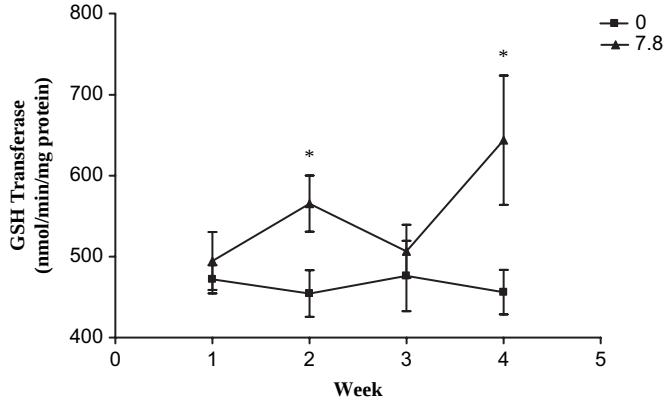

FIG. 5. Time-course study of activities of GSH *S*-transferase. Asterisk indicates significantly different from controls ($p < .05$, $n = 4–6$). Doses were mg/kg.

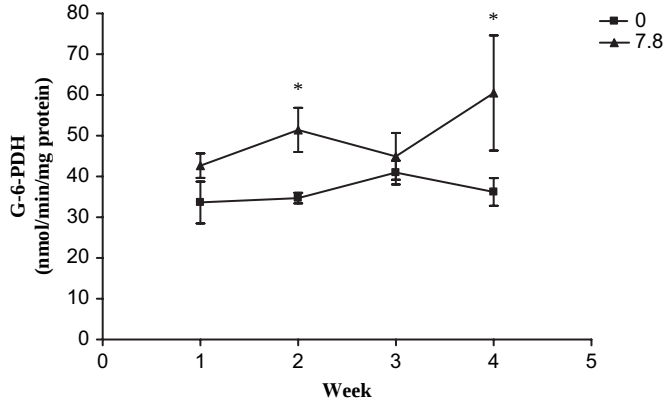

FIG. 6. Time-course study of activities of G-6-PDH. Asterisk indicates significantly different from controls ($p < .05$, $n = 4–6$). Doses were mg/kg.

DISCUSSION

In studies with birds and mammals, exposure to PCB 126 resulted in a variety of adverse effects (Fox & Grasman, 1999; Fairbrother et al., 2004). These include reduced growth, liver enlargement and necrosis, lymphocyte depletion with atrophy of associated organs, and histopathological alterations of the thyroid in nestling American kestrels (Hoffman et al., 1996a). Moreover, increased hepatic oxidative stress was reported following exposure to halogenated polycyclic hydrocarbons, including TCDD in Sprague-Dawley rats (Stohs et al., 1990), PCB 126 in mice (Hori et al., 1997), and PCBs in mink (Hoffman et al., 1992).

This study contributes to the existing literature on oxidative stress induced by coplanar PCBs or TCDD among vertebrate species. Several indications of altered glutathione metabolism

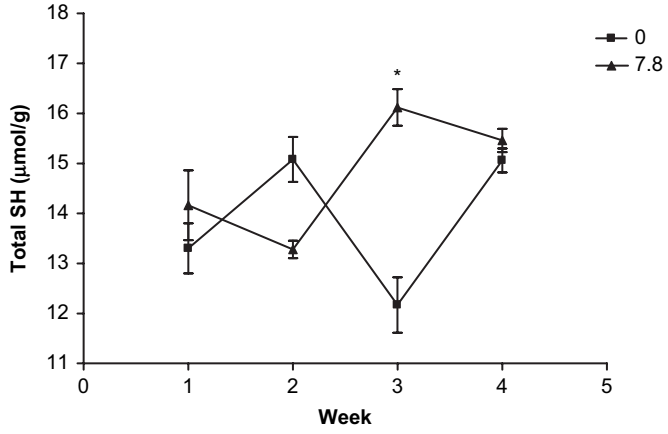

FIG. 7. Concentration of total SH. Asterisk indicates significantly different from controls ($p < .05$, $n = 4–6$). Doses were mg/kg.

and oxidative stress occurred. In the dose-response study, only glutathione peroxidase at the 2.3 mg/kg dose group was significantly lower than controls. TBARS did not show a dose-response curve like those of CYP450 enzyme activities. This phenomenon may be interpreted as a delayed oxidative stress which is manifested by the elevation of TBARS at wk 3 and 4 (see later discussion). The delay to reach maximal induction in CYP450 enzyme activities and elevated TBARS concentration was also observed by Palace et al. (1996) in juvenile lake trout following ip injections of PCB 126. Amphibians and fish are both ectothermic vertebrates, and we postulate that the delay found in enzyme induction in leopard frogs and fish may reflect their lower metabolic rate and physiological reactions at 20°C.

Except for glutathione peroxidase, the activities of GSSG reductase, G-6-PDH, and GSH *S*-transferase in the 7.8 mg/kg dosage group were higher than those in corresponding controls at similar points of time over 4 wk. These results indicate that these oxidative stress-related enzymes are inducible, presumably via aryl hydrocarbon receptors, in northern leopard frogs after exposure to coplanar PCBs. The predominant protective effect was the increase at wk 3 and 4 in the antioxidant, reduced glutathione. The increase of total SH is also probably a reflection of this. Though protective mechanisms were induced, the increase in TBARS suggests that toxicity occurred during the last half of the experimental period. In the future it would be interesting to correlate TBARS levels with cellular alterations in the liver. There also appears to be an increase, decrease, then recovery of activity of the enzyme G-6-PDH, which is responsible for generating NADPH, another protective antioxidant. In summary, the increase of activities with time relative to controls was indicative of both toxicity and the onset of protective mechanisms. Controls themselves appeared to be changing with time, perhaps due to age or physiological condition of frogs.

Although PCB concentrations have generally not appeared as high in amphibians as other vertebrates, there are some exceptions. Concentrations of up to 49.6 ppm whole-body PCBs were found in bull frogs (*Rana catesbeiana*) at a hazardous waste site in New York (Watson et al., 1985), up to 41.5 ppm wet weight in livers of green frogs (*Rana clamitans*) near a hazardous waste site in South Carolina (Fontenot et al., 2000), and up to 58.2 ppm wet weight was detected in ovarian tissues of mudpuppies (*Necturus maculosus*) from heavily contaminated sites along the St. Lawrence River of Ontario, Canada (Gendron et al., 1997).

Studies, including ours, on amphibian responses to polyhalogenated hydrocarbons indicate relatively low toxicity compared to other animals. Adult bullfrogs (*Rana catesbeiana*) collected along the 2, 3, 7, 8-TCDD-contaminated Rock Branch Creek in Arkansas with 2,3,7,8-TCDD levels ranging from 87 ng/kg in a muscle sample to 68,000 ng/kg in a fat sample were alive and appeared to be healthy (Korfmacher et al., 1986). Beatty et al. (1976) observed no significant mortality up to 50 d after ip injection of 1,000 μg/kg of 2, 3, 7, 8-TCDD into bullfrog tadpoles or up to 35 d after ip injection of up to 500 μg/kg into adult bullfrogs, and histopathological examination of various tissues such as liver, lung, and kidneys of the metamorphosed tadpoles and adult frogs failed to show any abnormalities. In contrast, studies on other animal species showed that single-dose oral LD_{50} values for 2, 3, 7, 8-TCDD ranged from 2 μg/kg for the guinea pig to 50 μg/kg for the monkey to 1,157 μg/kg for the hamster (Neal et al., 1982).

Conclusion

In conclusion, the present study revealed that altered glutathione metabolism and oxidative stress, indicative of both toxicity and induction of protective mechanisms, occurred as a delayed response in frogs exposed to a relatively high concentration of a particularly toxic PCB congener. It is of particular interest to note that delayed toxicity such as this may occur in amphibians, unlike more immediate manifestations occurring in warm blooded vertebrates including mammals and birds. Therefore, duration of exposure observation time should be taken into consideration when designing amphibian toxicity studies.

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