IS OXIDATIVE STRESS THE MECHANISM OF BLUE SAC DISEASE IN RETENE-EXPOSED TROUT LARVAE?

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Abstract—Retene (7-isopropyl-1-methylphenanthrene) causes blue sac disease (BSD) in early life stages of fish, an effect similar to that of 2,3,7,8-tetrachlorodibenzo( p)dioxin. The signs of BSD include cytochrome P450 (CYP1A) induction, edema, hemorrhaging, and craniofacial deformities, indicating membrane damage, circulatory failure, and impaired development. To test if the underlying cause was oxidative stress, rainbow trout (Oncorhynchus mykiss) larvae were exposed to waterborne retene or to known prooxidants (paraquat, t-butyl hydroperoxide, and carbon tetrachloride) in the presence or absence of vitamin E, an antioxidant. Fish exposed to retene showed an increased prevalence of BSD, reduced tissue concentrations of vitamin E and total glutathione, and a lower percentage of glutathione in a reduced form. Coexposure to vitamin E reduced the prevalence of BSD and restored tissue concentrations of vitamin E, but it did not affect retene uptake or tissue concentrations of glutathione. These responses are consistent with oxidative stress as a mode of action of retene. However, retene did not affect whole-body lipid peroxide concentrations, and prooxidants did not affect the prevalence of BSD and had only minimal effects on tissue glutathione and vitamin E. Possible explanations for these conflicting results include prooxidant exposures were insufficient to generate oxidative stress; lipid peroxidation may not be measurable in whole-body homogenates of retene-exposed fish if effects are localized to endothelial cells, where CYP1A enzymes are most induced; or retene may have an alternate mode of action (e.g., adduction of retene metabolites to lipids, protein, or DNA).

Keywords—Fish Polynuclear aromatic hydrocarbons Retene Oxidative stress Early life stage

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are found in crude oil, creosote, coal tar, and aquatic sediments near industrial discharges. Concerns for their environmental effects have focused traditionally on the U.S. Environmental Protection Agency’s 16 priority PAHs, most of which have no alkyl side chains. However, in complex hydrocarbon mixtures, such as crude oil, more than 95% of total PAHs may be alkyl-substituted [1]. The importance of alkyl-substitution was recognized when mortality in early life stages of Pacific herring (Clupea pallasi) and pink salmon (Oncorhynchus gorbuscha) followed contamination of spawning shoals in Prince William Sound (AK, USA) by oil spilled from the Exxon Valdez. Mortality was associated with an elevated prevalence of blue sac disease (BSD), the severity of which could be correlated to concentrations of alkyl-substituted PAHs, such as the alkyl-phenanthrenes [2]. The occurrence of BSD was replicated in the laboratory by exposure of fish embryos and larvae to Alaskan North Slope crude oil (salmon) [2] or to retene (7-isopropyl-1-methyl phenanthrene; trout) [3]. The signs of BSD closely resembled those caused by tetrachlorodibenzo-p-dioxin (TCDD) and included hemorrhaging, pericardial and yolk sac edema, craniofacial deformities, and induction of cytochrome P450 proteins (specifically CYP1A) in the vascular endothelium of gill, kidney, heart, and other tissues [3,4].

Induction of CYP1A refers to activation of the cypla gene and the increased synthesis of CYP1A protein following binding of PAHs to the arylhydrocarbon-receptor (AhR) protein. The CYP1A protein is a catalyst for many monoxygenation reactions, and retene can be hydroxylated to benzylic alcohols by this enzyme system [5]. Oxygenation of PAHs often is the first step in their metabolism and subsequent excretion, but oxygenation of retene may contribute to the development of BSD. Billiard et al. [3] proposed an oxidative stress model in which sustained induction of CYP1A enzymes resulting from continuous exposure to retene produces an excess of reactive oxygen species (ROS). Typically, ROS are produced in low quantities as part of basal metabolism and are neutralized by a variety of cellular antioxidants. However, when produced in large quantities, ROS can overwhelm oxidative stress defenses and damage proteins, lipids, and DNA. Lipid peroxidation is a self-propagating reaction that, if unchecked, can damage the endothelial membranes of the vascular system and cause edema and hemorrhaging (for review, see [4,6,7]).

If this model is correct, lipid peroxidation should increase, and concentrations of antioxidants should decrease, in fish larvae that show signs of BSD following exposure to retene. Other compounds known to produce oxidative stress (i.e., prooxidants) should similarly cause BSD. Finally, coexposure of larvae to retene and to an antioxidant should reduce the prevalence of BSD.

The role of oxidative stress in retene toxicity was evaluated by measuring biochemical indicators of oxidative damage (tissue concentrations of glutathione, vitamin E, and lipid peroxides [LPOs]) and the prevalence of BSD in larvae of rainbow trout exposed to waterborne retene. To determine if the prevalence of BSD could be reduced by an antioxidant, larvae were coexposed to retene and to a range of waterborne vitamin E concentrations. To determine if BSD was a general response...
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Both PQ and t-B cause lipid peroxidation and changes in glutathione metabolism of exposed fish [8–10]. Carbon tetrachloride is hepatotoxic, and its effects are associated with metabolism by cytochrome P4502E1 (CYP2E1), one of several cytochrome P450 enzymes that act on CCl4. The mechanism may involve lipid peroxidation because antioxidants and P450 inhibitors reduce CCl4 toxicity, whereas vitamin E deficiency increases susceptibility [11,12]. However, the role of peroxidation in CCl4 tissue damage is not clear.

Reduced glutathione (GSH) is an important cellular antioxidant, and the ratio of reduced to oxidized glutathione (GSSG) forms has been used as a reliable indicator of cellular redox status. Reduced glutathione also is conjugated to xenobiotics by glutathione-S-transferase enzymes in the second step of detoxification (phase II) [11]. It is also an antioxidant, reacting with aldehydes produced by lipid peroxidation and, thereby, protecting -SH groups of membrane proteins [10]. As an antioxidant, it is oxidized to GSSG during the reduction of hydrogen peroxide and organic peroxides (including LPOs) to alcohols by glutathione peroxidase [9,13]. If retene causes oxidative stress, the proportion of total glutathione (Gp) in the reduced form should decrease.

In vitro, rainbow trout microsomes were resistant to lipid peroxidation relative to rat and rabbit microsomes, and resistance was associated with a comparatively high concentration of vitamin E [14]. However, trout embryos and larvae only have finite supplies of maternally derived antioxidants in yolk reserves so that endogenous vitamin E may become depleted during retene exposure [3,7]. Cotreatment with exogenous vitamin E might reverse this depletion and reduce the prevalence of BSD.

MATERIALS AND METHODS

Design

Newly hatched trout larvae were exposed to test compounds at concentrations known to be toxic to fish using a static daily-renewal protocol. Toxic concentrations were selected to ensure that signs of oxidative stress would be observed should oxidative stress be the mode of toxicity. The test concentrations were as follows: Retene, 320 μg/L, after Billiard et al. [3]; PQ, 1 mg/L, after Fytizas [15] and Sinhaseni and Tesprateep [16]; t-B, 9 mg/L, approximately 10% of the 24-h median lethal concentration (LC50); J. Meyer, Duke University, Durham, NC, USA, personal communication); and CCl4, 8 mg/L, after Statham et al. [17]. Retene causes BSD at 320 μg/L in static daily-renewal tests [3] and at 9 μg/L in continuous-flow tests [5]. Because the objective was to induce toxic effects and not to describe threshold effect concentrations, the daily renewal protocol was selected for its simplicity.

Four tanks were spiked with each test compound, with the first receiving the compound only and the remaining three receiving the compound plus waterborne vitamin E at concentrations of 0.1, 1, or 10 mg/L. A range of vitamin E concentrations was tested, because to our knowledge, no previous information about effective waterborne concentrations has been reported. Three control tanks received vitamin E only at 0.1, 1, or 10 mg/L, and a negative control tank received 300 μL of isopropanol, the solvent carrier for retene and vitamin E. This concentration (~30 mg/L) is well below the 96-h LC50 of 11,130 mg/L [18]. Both PQ and t-B solutions were administered as aqueous solutions, and CCl4 was added directly.

Fertilized rainbow trout eggs at the eyed stage (Rainbow Springs Trout Hatchery, Thamesford, ON, Canada) were maintained in the dark at 6.3 to 8.5°C in aerated, dechlorinated Lake Ontario water (alkalinity, 120 mg/L; pH 7.8–8.0) supplied by the City of Kingston (ON, Canada). At the first sign of hatching (day 0 of the experiment), batches of 45 unhatched eggs were placed into the 4-L jars with 3 L of 7.8°C water spiked with test chemicals. Hatch in all treatments followed within 24 to 48 h so that the majority of the exposure period occurred during the larval stage. Treatment solutions were renewed daily by decanting all liquid except for the last few milliliters covering the eggs, and a fresh solution was added immediately. Temperature was adjusted over the first 4 d to 10°C, after which it was monitored daily and ranged from 8.3 to 11.5°C. Dissolved oxygen was measured every second or third day and ranged from 8.3 to 13.7 mg/L, and pH was measured on days 5 and 12 and ranged from 8.10 to 8.39. Mortality was recorded daily throughout the experiment, and dead eggs or larvae were removed.

The experiment was terminated just before swim-up when retene-exposed larvae showed signs of BSD to an extent that mortality was imminent. Surviving larvae were anesthetized with tricaine methane sulfonate (MS-222; 100 mg/L), examined by dissecting microscopy to score signs of BSD, and assigned sequentially to different biochemical assays without regard to whether they showed signs of BSD. Fish for glutathione analysis (n = 10 per tank; n = 8 analyzed per tank) were blotted dry, weighed, and homogenized immediately (as described below). Larvae for vitamin E (n = 10 per tank) and LPO (n = 10 per tank; n = 6–7 analyzed per tank) analyses were blotted dry, placed in 1.0-ml cryogenic vials, frozen in liquid nitrogen, and stored at −80°C. For immunohistochemical localization of CYP1A protein, 10 larvae/tank were transferred to Bouin’s fixative for 24 h and then to 70% ethanol for storage before five fish per tank were processed.

Waterborne retene

Exposure solutions were sampled daily, diluted 1:1 (v/v) with high-performance liquid chromatography (HPLC)–grade ethanol, and stored in the dark at 4°C until analysis. Before analysis, samples were sonicated for 5 to 10 min at less than 30°C. Retene concentrations were measured by synchronous scanning fluorescence at an excitation–emission wavelength offset of 52 nm using a fluorescence spectrometer (Quantum Master 1) with a 75-W xenon arc-lamp, no infrared filter, and FELIX software (Photon Technologies International, London, ON, Canada) [4,19]. The area under the fluorescence spectrum was integrated to calculate retene concentrations from a standard curve for retene (4–300 μg/L).

Blue sac disease

The signs of BSD scored were craniofacial deformities; yolk sac and pericardial edema; hemorrhaging in the cranial, ocular, and yolk sac regions; and caudal fin erosion [3]. Craniofacial deformities included a gaping jaw, blunted snout region, and a deformed and exaggerated dome shape of the cranium. Other deformities or areas of hemorrhaging occasionally were observed but were not included in the score. For each treatment or control, the number of fish showing each sign was counted and the final score given as the total number of
BSD signs divided by the number of fish per tank. When rates of BSD were elevated (i.e., in retene treatments), virtually all fish showed one or more signs so that final scores were higher than a simple count of affected fish but not inflated by a few fish showing severe responses.

**Lipid peroxide**

Lipid peroxide concentrations were measured with a commercial kit (Kamiya Biomedical, Seattle, WA, USA) that is specific to LPOs and unaffected by other aldehydes in the assay, a concern with the thiobarbituric acid–reactive substances (TBARS) assay that measures malondialdehyde [20]. The LPO assay uses hemoglobin to facilitate the reduction of lipid hydroperoxides to hydroxyl derivatives, while concurrently, a colorless chromogen (10-N-methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine) is oxidized to methylene blue. The amount of methylene blue formed is measured colorimetrically at 675 nm [20].

Whole larvae from each tank were homogenized individually in 20 volumes (e.g., 100 mg of tissue in 2.0 ml) of 2:1 (v/v) chloroform:methanol containing 0.04% (w/v) of butyrylated hydroxytoluene. Samples were mixed with 600 μl of 0.9% saline and centrifuged at 4.0°C for 10 min at 3,000 g to create organic and aqueous phases. An aliquot of 500 μl of the organic layer was dried by vacuum rotary evaporation, and the pellet was resuspended in 300 μl of isopropanol.

Aliquots of 7.0 μl of standard, blank, and sample solutions were plated in triplicate on a 96-well microplate with 70 μl of the LPO kit’s Reagent 1, followed by 140 μl of the kit’s Reagent 2 after 5 min of incubation at 30°C. Bubbles were removed by octanol fumes. The plate was incubated for 10 min at 30°C and mixed, and the absorbance was measured at 675 nm with a SPECTRAmax® PLUS microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All results fell within the linear range of a standard curve of cumene hydroperoxide (2.0–300 nmol/ml) [20].

**Glutathione**

Freshly sampled larvae were rinsed in ice-cold 0.15 M KCl, blotted dry, weighed, and homogenized with five volumes of ice-cold 5.0% (w/v) 5-sulfosalicylic acid dihydrate. The homogenate was centrifuged at 10,000 g at 4°C for 10 min, and the supernatant was frozen in liquid nitrogen and stored at −80°C.

Glutathione concentrations were measured by a modification of method described by Baker et al. [23]. Reduced glutathione was oxidized to GSSG by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and recycled back to GSH by glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) [10,24]; two molecules of GSH are regenerated from one molecule of GSSG, the dimer. The rate of formation of 5-thio-2-nitrobenzoic acid, which is produced in the reaction of GSH with DTNB, was monitored at 405 nm [23]. The assay incorporated separate procedures for GSSG, a dimer of glutathione, and for Gp. Total glutathione represents oxidized and reduced forms, which are combined as glutathione equivalents [23]. The amount of GSH was determined by the difference between Gp and GSSG in each sample.

For GSSG, aliquots of supernatant and standard solutions were incubated with 2-vinylpyridine (2-VP; 2 μl) to remove GSH and to leave GSSG as the form of glutathione available for DTNB reduction [23,24]. For Gp, all assay procedures were the same as those used for GSSG, except that no incubation with 2-VP was used. Because GSSG (dimer) and GSH (monomer) reduce DTNB at the same rate, GSSG standard solutions (0 to >1,000 pmol/well) were prepared from GSSG (purity, ≥98%; Sigma Chemical, St. Louis, MO, USA) [23].

Reaction mixtures were fresh solutions of 1.0 mM β-NADPH, 1.0 mM DTNB prepared in buffer (pH 7.5; 100 mM NaHPO₄·H₂O and 10 or 13 mM ethylenediaminetetraacetic acid), and sufficient glutathione reductase (from Bakers yeast; Sigma Chemical) to provide 1.0 U/ml of reaction mixture. The nonenzymatic reaction components were mixed in a ratio of 23% NADPH, 31% DTNB, and 45% buffer. For GSSG and Gp analyses, 100 μl of tissue supernatant (plus 2 μl of 2-VP for GSSG) were mixed for 1.0 min with 20 μl of 10% (v/v) triethanolamine in phosphate-buffered saline (PBS pH 7.4; 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl) and incubated on ice in the dark for 1.0 h. Triplicate samples of 10 μl were pipetted onto a 96-well microplate, followed by 200 μl of the reaction mixture. The wells were mixed for 10 s, and the absorbance was recorded for 2.0 min at 405 nm with a SPECTRAmax® PLUS microplate spectrophotometer. The maximum velocity of the reaction (Vₘₕₜ) was calculated by a quadratic curve fit.

Both Gp and GSSG concentrations were measured directly, whereas GSH (the difference between GSSG and Gp), the ratio of GSH to Gp, and the ratio of GSH to GSSG were calculated.

**Vitamin E**

Vitamin E was measured by a modified version of the reverse-phase HPLC method of Palace et al. [25]. Within a treatment, frozen larvae were thawed, pooled in groups of two, weighed, and homogenized in ice-cold distilled water for 1.0 min. The homogenate was mixed with an internal standard of 58 μg of dextro-levorotatory (DL)-α-tocopherol acetate (Supelco, Supelco Park, Bellefonte, PA, USA) in 1.0 ml of HPLC-grade ethanol (to precipitate proteins).

To extract vitamin E, 2.0 ml of HPLC-grade ethyl acetate:hexane solution (3:2 [v/v]; Fisher Scientific, Fair Lawn, NJ, USA) were added, mixed, and let stand at room temperature in the dark for 15 min, with remixing every 5 min. Homogenates were centrifuged for 3 min at 10,000 g, and 1.0 ml of the supernatant (organic solvent) was concentrated in a vacuum rotary evaporator in the dark. Concentrates were resuspended in 250 μl of HPLC mobile-phase solution and stored at −80°C until analysis.

Concentrates were analyzed by a Prostar® HPLC and autosampler (Varian Chromatography Systems, Mitchell Drive/ Walnut Creek, CA, USA). The mobile phase was 70:20:10 acetonitrile:chloromethane:methanol (v/v/v) with 1% propionic acid; all solvents were HPLC grade (Fisher Scientific). The flow rate of the mobile phase was 1.0 ml/min through a Zorbax ODS analytical column (length, 250 mm; inner diameter, 4.6 mm; pore size, 5.0 μm; Chromatographic Specialties, Brockville, ON, Canada). protected with a 5-μm screen and a Zorbax ODS guard column. Tocopherol was measured at 292 nm against an eight-point standard curve of DL-α-tocopherol acetate in ethanol; the mobile phase alone served as a blank. Sample results were corrected for percentage recovery of the internal standard, which averaged 75.5% ± 1.5% (95% confidence limit, n = 99).

**CYP1A concentrations**

To verify retene exposures and the co-occurrence of CYP1A induction and BSD, CYP1A protein was measured semiquan-
titatively by immunohistochemistry following the method described by Smolowitz et al. [26], with modifications as described by Brinkworth et al. [4]. Individual larvae were fixed in Bouin’s fixative, stored in 70% ethanol, embedded in wax, sectioned (thickness, 5.0 μm), and mounted on glass slides. Liver tissue of juvenile trout exposed to β-naphthoflavone or to water-only served as positive and negative controls, respectively.

Tissue sections were dewaxed with xylene and rehydrated with successive immersions in 100, 95, and 70% ethanol, 3% hydrogen peroxide, water, and 1% bovine serum albumin (BSA) in PBS. Slides were stained with purified mouse monoclonal antibody (MAb) 1-12-3 against scup CYP1A as the positive antibody (donated by John Stegeman, Woods Hole Oceanographic Institute, Woods Hole, MA, USA) using the ULTRA PAP Detection System (ID Labs, London, ON, Canada) with successive immersions in 100, 95, and 70% ethanol, 3% hydrogen peroxide, water, and 1% bovine serum albumin (BSA) in PBS. Slides were stained with purified mouse myeloma immunoglobulin (IgG1, κ) produced from mineral oil induced plasmacytoma cells (MOPC 321; Sigma Chemical) diluted to 0.3 μg/ml in 1% BSA/PBS.

Slides in disposable immunostaining chambers (Fisher Scientific Canada, Nepean, ON, Canada) were covered with 2.0 ml of BSA/PBS. A 5-min incubation with 150 μl of 5% normal goat serum (blocking reagent) was followed by two 1-h incubations with 150 μl of either positive or negative antibody and a 2.0-ml wash with BSA/PBS. Slides were incubated with goat antimouse immunoglobulin (Ig) G (linking reagent, 150 μl) for 20 min, washed with BSA/PBS, incubated with 150 μl of peroxidase-linked mouse IgG (labeling reagent) for 20 min, and washed with 2.0 ml of BSA/PBS followed by 2.0 ml of PBS alone. Color was developed by two 10-min incubations with 150 μl of freshly prepared 3-amino-9-ethylcarbazole substrate chromogen in substrate buffer (50:1 v:v), followed by three water washes. Sections were counterstained with hematoxylin, washed, rinsed five times in 0.56% NH₄OH, and washed again, and then coverslips were added.

Cytochrome P450 protein concentrations in larval kidney were scored by microscopy [27]. The score (0–15) was the product of staining frequency (0 = no cells stained, 1 = rare or few cells stained, 2 = multifocal staining, and 3 = diffuse staining) multiplied by intensity (1 = very mild, 2 = mild, 3 = moderate, 4 = strong, and 5 = very strong). All slides (except for positive controls) were coded and scored “blind” at a fixed magnification and lighting intensity; none of the negative controls showed staining.

**Data analysis**

Results were tested for statistical significance at \( p < 0.05 \) with SigmaStat for Windows (Ver 1.0; Jandel, San Rafael, CA, USA) using a two-way analysis of variance (ANOVA). Differences among means were identified by the Student-Newman-Keuls test unless otherwise indicated in figure captions. Calculations were performed, and critical \( t \) values were obtained from those reported by Zar [28]. Based on tests of normality and homogeneity of variance, LPO data were log-transformed before analysis, but all other data were untransformed. On all graphs, error bars represent 95% confidence limits as calculated from the pooled error variance of the ANOVA, the sample size for each treatment, and Student’s \( t \) statistic for error degrees of freedom. Using the pooled error variance from the ANOVA as the best estimate of the true variance means that the error bars will appear to be equal among treatments when sample sizes are equal.

This experiment originally was designed for multiple linear-regression analysis to relate effects of chemical treatments (coded as dummy variables) to effects of different concentrations of vitamin E. The design was replicated only once, assuming that prooxidant effects coupled with variable levels of vitamin E would define a response surface. Because prooxidants had few effects on the parameters measured (i.e., no change from control), the ANOVA of main effects was substituted for the multiple regression. Tests of main effects pooled the prooxidant effects across all vitamin E concentrations and vitamin E effects across all prooxidant exposures.

For all treatments except retene, scores for CYP1A staining and signs of BSD were the same as those of control larvae exposed to isopropanol and vitamin E. Hence, data for all treatments except retene were pooled, means and 95% confidence limits calculated, and results for retene-exposed larvae compared to the pooled results to determine if they would be considered as part of the same population of responses.

**RESULTS**

**Retene concentrations in water**

Within 24 h of dosing, average measured retene concentrations in tank water declined to 68 to 89% of the nominal concentration of 320 μg/L, which is consistent with the findings of Billiard et al. [3], who observed declines to 60 to 67% of nominal because of partitioning to tank walls and to fish.

**Mortality**

Mortality of larvae was less than 10% in all treated and control tanks except for fish exposed to CCl₄, for which mortality ranged from 12 to 20%. Mortality was not analyzed statistically because of bias created by sampling all treatments when mortality rates of retene-exposed fish appeared to increase near swim-up (i.e., after ~16 d of exposure at 10°C [157 degree-days]).

**BSD and CYP1A scores**

For both BSD and CYP1A scores, values for the single retene treatments were consistently 8- to 20-fold higher than pooled means for all other treatments and well outside their 95% confidence limits (Fig. 1). However, BSD scores were approximately one-third lower in retene-treated fish coexposed to 10 mg/L of vitamin E relative to fish exposed to retene alone (Fig. 1B). In contrast, the CYP1A score was unaffected by coexposure to vitamin E (Fig. 1A).

**Lipid peroxidation**

No significant differences in concentrations of whole-body LPO concentrations were observed among any prooxidant or vitamin E treatments (data not shown). Means ranged from 0.25 to 0.42 nmol/mg wet tissue (95% confidence interval, ±0.13 nmol/mg), and no systematic trends were noted in LPO concentrations with vitamin E concentration.

**Glutathione**

The concentrations of Gp, the proportions that were GSH, and the ratios of GSH to GSSG were consistently lower in retene-treated larvae relative to the vitamin E controls and all other treatments (Fig. 2). Carbon tetrachloride caused a slight (12%) increase in Gp above the level in the vitamin E control.
Retene decreased the GSH to GSSG ratio by almost 70% from that in the vitamin E control (Fig. 2C), but the other prooxidants decreased the ratio by only 30 to 35%.

Vitamin E exposure had no effect on Gp concentrations or on the GSH to Gp ratio (data not shown), either alone or in combination with prooxidants. However, vitamin E alone or vitamin E plus PQ or CCl₄ caused statistically significant changes in GSH to GSSG ratios, but these changes were not exposure-dependent (Fig. 3).

**Vitamin E**

Retene exposure caused a significant decrease (20%) in whole-body vitamin E concentrations relative to those in the vitamin E control and other treatments (Fig. 4), and concentrations were restored to control levels by coexposure to 10 mg/L of vitamin E (Fig. 5). This interaction was only observed for retene, and none of the other prooxidants reduced tissue vitamin E concentrations. Vitamin E concentrations caused increased tissue vitamin E concentrations (Fig. 4), which were consistent across all retene and prooxidant treatments (Fig. 5).

**DISCUSSION**

The present study demonstrated that exposure during early life stages of rainbow trout to retene caused CYP1A induction, a greater prevalence of BSD, and decreased concentrations of tissue antioxidants (GSH and vitamin E). Concentrations of tissue LPOs were not elevated by retene, but the prevalence
that retene, a compound that can bind to the AhR-receptor protein [29], was being taken up; no induction occurred in larvae exposed to other prooxidants. High CYP1A scores were associated with a high prevalence of BSD, and only retene-treated fish had a greater prevalence of BSD than that of controls. Although signs of BSD were scored using a dissecting microscope, some signs were sufficiently severe to be visible to the naked eye, and in retene treatments, virtually all fish showed one or more signs. The co-occurrence of CYP1A induction and BSD correspond to observations by Brinkworth et al. [4], who found that CYP1A protein concentrations in retene-exposed trout larvae increased immediately after hatch, in parallel with subsequent increases in the prevalence of BSD.

The retene-induced decreases in tissue antioxidants and the reduction in prevalence of BSD with co-exposure to vitamin E are consistent with oxidative stress as a mode of action. In this model, oxidative stress would follow retene accumulation, CYP1A induction, and an accelerated output of ROS when retene is metabolized by CYP1A enzymes.

In contrast, exposure to model prooxidants did not cause BSD in larval fish, possibly because exposures were too low or their modes of action differed from that of retene. We chose exposure concentrations to generate chronic, sublethal toxicity, corresponding to the observed action of retene. For CCl4 at 8 mg/L, mortality rates were approximately 10% above those of other treatments, indicating chronic toxicity; at 80 mg/L, 100% mortality occurred (data not shown). For PQ and t-B, mortality was the same as that in controls, and we had no method for measuring waterborne concentrations. Hence, we cannot be certain that chronically toxic exposures were attained.

In vitro, lipid peroxidation appears to be an important mechanism by which low concentrations of t-B kill cells in acute exposures [30]. However, reports of oxidative stress in live fish are characterized by acute doses of injected prooxidants and transient effects. Trout injected with PQ showed elevated concentrations of soluble TBARS or plasma LPOs after 24 to 48 h [8,31] when exposures were sufficient to cause histopathology in several tissues [15]. To observe significant changes in tissue concentrations of Gp and increased TBARS, Plöch et al. [10] had to pretreat juvenile catfish with diethylmaleate to deplete Gp stores before injecting 40 mg/kg of t-B. Even under these severe conditions, tissue concentrations of Gp and TBARS began to recover within 48 h, with maximal increases in TBARS of two- to fourfold by 12 h postexposure; no significant changes in concentrations of oxidized DNA were observed [10]. Hence, these prooxidants may induce oxidative stress in fish only after acute exposures, and larval trout may have sufficient antioxidant capacity to prevent oxidative stress during chronic, sublethal exposures.

Lipid peroxidation can accompany acute PQ exposure. However, it is not the main mediator of PQ-induced damage, although PQ toxicity involves redox cycling and production of superoxide anion [9] (for review, see Smith [32]). For CCl4, the liver is the major organ damaged [10], and the pathology in trout is similar to that noted in mammals [17]. Activation of CCl4 to reactive metabolites is carried out most effectively by CYP2E1 [12], not by CYP1A. In fact, low CYP1A staining in larvae exposed to CCl4 suggests inhibition of CYP1A induction, which is consistent with the observed inhibition of CYP1A enzymes [33]. In contrast, BSD caused by retene and by dioxin-like compounds is localized within vascular endothelial cells and is associated with CYP1A induction, vascular damage, and impaired circulation [3,34,35], not with liver pa-
thology. Hence, the production of oxyradicals by vertebrates exposed to dioxin like-compounds, such as 3,3',4,4'-tetrachlorobiphenyl (TCB) [36], may be unique, because prooxidants do not induce CYP1A enzymes and do not generate ROS by CYP1A activity. For readily metabolized alkyl-PAHs, such as retene, toxicity also might be caused by reactive metabolites [5].

The oxidative stress model predicts an increase in lipid peroxidation within the vasculature because of excess ROS production by increased CYP1A enzyme activity. However, while the concentrations of plasma LPOs increased in juvenile rainbow trout injected with PQ [8] and in the liver of different populations of adult lake trout [37], the LPO assay showed no response in whole-larvae homogenates of any treatment groups in the present study. Either oxidative stress did not occur in larvae exposed to prooxidants, in contrast to juvenile trout [8], or the LPO method was inappropriate for these samples. We have found no other publications reporting LPO assays on larval whole-body homogenates, so to our knowledge, no equivalent data are available for comparison. False negatives might arise from chemical interference with the method by components of the yolk or from changes in LPO concentrations within vascular endothelium that were too small to detect in whole-body homogenates. The first alternative cannot be evaluated, because the reagents in the LPO kit are proprietary.

The absence of lipid peroxidation also might be correct. Rainbow trout appear to be resistant to lipid peroxidation even when depleted of the antioxidant glutathione [8], and vitamin E may provide a defense against lipid peroxidation in vivo. In vitro, vitamin E plays a key role in preventing lipid peroxidative damage [14], because it is embedded in membranes, making it efficient at terminating peroxidation chain reactions [38]. A main effect of retene treatment was a 20% decrease in whole-body vitamin E concentrations relative to the isopropanol and vitamin E control groups (Fig. 4). Whole-body vitamin E concentrations for isopropanol control larvae were slightly lower, but still comparable, to those reported for lake trout larvae [37]. In fish exposed to retene plus 10 mg/L of vitamin E, a complete restoration of tissue vitamin E concentration and a decrease in the prevalence of BSD were observed, suggesting a strong interaction between the modes of action of retene and vitamin E. This interaction could be either an antioxidant effect of vitamin E or a reduction in exposure to retene, because both compounds are hydrophobic. However, CYP1A staining did not vary with vitamin E exposure (Fig. 1), indicating that vitamin E did not affect retene accumulation. Unlike retene, prooxidants had no significant effects on vitamin E concentrations relative to those in the isopropanol and vitamin E control groups.

Retene exposure was associated with a 25% decrease in whole-body Gp concentration compared to the isopropanol and vitamin E control groups. The antioxidant form, GSH, accounted for 90% of Gp in the control group, but it comprised only 78% of Gp in retene-treated larvae. Vitamin E coexposure, at any dose used in the present study, did not affect Gp or the ratio of GSH to Gp among any of the retene treatments, which might indicate that glutathione plays a role in oxidative stress defense at a stage other than lipid peroxidation. Depletion of Gp also might reflect high rates of retene excretion as glutathione conjugates.

An initial decrease in cellular glutathione also may be an important upstream signal in a pathway of vascular endothelial cell stress response and apoptosis caused by TCB [39]. Similarly, the embryotoxicity of TCDD appears to involve apoptotic cell death in the embryonic vasculature, leading to defective nutrient transport from the yolk sac to the embryo [32,40]. Slim et al. [39] proposed that endothelial cells might be susceptible to polychlorinated biphenyl (PCB)-induced injury, because PCBs and hydroxylated PCB metabolites persist in the blood for some time. In juvenile rainbow trout, retene has a calculated half-life of 14 h, but elevated concentrations in tissue can be maintained by continuous exposure to waterborne retene [41]. Low concentrations of retene also have been found in tissues of larval rainbow trout six weeks after they were removed from retene exposure [4]. A common toxic mechanism proposed for PAHs and chlorinated PAHs includes apoptosis [3]. Persistence of retene during early life stages might contribute to glutathione depletion in larvae, and as with TCB [39], this depletion might trigger apoptosis-signaling events in vasculature endothelial cells.

Paraquat, t-B, CCl₃, and particularly, retene all had the main effect of significantly decreasing the ratio of GSH to GSSG relative to that in the isopropanol and vitamin E control group. This change in the redox forms of glutathione suggests the potential for oxidative stress, but it should be interpreted with caution. Cowey et al. [7] found that glutathione S-transferase and glutathione peroxidase enzyme activities were very low in fertilized Atlantic salmon (Salmo salar) eggs but increased considerably at hatch. Concurrent with these increases in enzyme activities was an increase in the amount of GSH needed for the reactions catalyzed by these enzymes [7], presumably to enhance antioxidant defenses after hatch [7]. In salmon, concentrations of vitamins E and C dropped from the eyed-egg through larvae to swim-up stages, with vitamin C reaching especially low values [7]. Brinkworth et al. [4] suggested that during early life stages of trout exposed to retene, hatch might be a period when antioxidant defenses are increasingly strained. Shortly after hatch, CYP1A concentrations increased dramatically, presumably because the protective chorion was removed and retene uptake increased. Assuming that similar events occurred in the present study, the decrease in vitamin E and Gp because of retene exposure probably increased the depletion of finite antioxidant reserves that are derived maternally [3,7]. Thus, it appears that sensitivity to retene-induced oxidative stress may be particularly high between hatch and swim-up because of increasing aerobic metabolism and production of ROS [4,7,42], upregulated glutathione-dependent defenses [7] that may exceed the capacity to synthesize glutathione [9], and normal depletion of finite antioxidant supplies until initial feeding [7].

In summary, we measured biochemical indicators of oxidative stress in tissues of larval trout exposed to retene and to prooxidants to determine whether oxidative stress is a logical explanation for the mechanism of retene toxicity. Retene-exposed trout showed CYP1A induction, depletion of tissue antioxidants, and a greater prevalence of BSD that was reduced with vitamin E coexposure. Although these results are consistent with an oxidative stress mechanism, some contradictions were observed: Retene effects were not mimicked by exposure to known prooxidants, and retene did not cause increases in whole-body LPO concentrations. Published reports of oxidative stress by prooxidants have always involved acute exposures, and the concentrations used in this chronic, sublethal test may have been inadequate to overwhelm the oxidative defense system of trout. As well, the whole-body assay of LPO concentrations may have been inappropriate if CYP1A
metabolism of retene generates oxiradicals in peripheral vasculature. Further tests of the oxidative stress model require improved methods for localizing production and effects of ROS at the cellular level, verification that prooxidants do not cause oxidative stress in larval fish, and investigation of the reactive metabolites of retene that might form adducts with lipids, proteins, or DNA.

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