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Cytochrome P450 1A- and stress protein-induction in early life stages of medaka (*Oryzias latipes*) exposed to trichloroethylene (TCE) soot and different fractions

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It has previously been shown that trichloroethylene (TCE) soot extracts cause dioxin-like toxic effects in medaka fish (*Oryzias latipes*) and primary liver cell culture of rainbow trout (*Oncorhynchus mykiss*). This study examines embryonic and larval induction of cytochrome P450 1A and stress proteins after exposure of medaka embryos to extracts and fractions of TCE combustion-generated aerosols. Embryos were exposed to three concentrations of whole soot extract (WE; 2.7, 7.2 and 18 μg l^{-1} incomplete combustion by-products), TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3 ng l^{-1}) and four TCE fractions with different polarity (Fr 1–4; 18 μg l^{-1}) for 8 days. Approximately 50% of the embryos were then transferred to control water and allowed to hatch. EROD activity in embryos was significantly higher than in controls after the 8 day-exposure to TCE soot extract (WE), with activity being highest at 2.7 μg l^{-1} WE (5.6× control). Of TCE fractions, only fraction 1 (Fr1, non-polar compounds) caused a significant increase in EROD activity. In larvae, significantly induced EROD activity was detected following the 7.2 μg l^{-1} WE treatment (3.30 pmol min^{-1} mg prot. $^{-1}$). Dioxin treatment did not result in increased embryonal or larval EROD activity. Larval CYP 1A was localized mainly in liver, gut, kidney, cornea and chondrocytes of cranium and tail. Hsp70 was induced in larvae but not in embryos. Statistically significant induction over controls was observed in two WE groups (2.7, 7.2 μg l^{-1}) and in the group exposed to dioxin (WE 18 μg l^{-1} not analysed). Mean hsp60 levels were not significantly higher than controls. Apparent bacterial contamination may have induced hsp70 in one control group including embryos and larvae (C/Fr3).

Keywords: cytochrome P450 1A, hsp60, hsp70, medaka (Oryzias latipes) trichloroethylene

Introduction

Incineration has been widely used as a means for disposal of municipal, hospital and industrial hazardous wastes. Its use has been curtailed in recent years because of concern about the emission of toxic byproducts associated with soot particles, especially chlorinated phenols, aromatic hydrocarbons, polychlorinated dibenzodioxins, and dibenzofurans (Seeker 1990, Lafleur *et al*. 1993, Wendt 1994). These emissions arise from improper operation of incinerators or from transients (Linak *et al*. 1987a,b, Wendt and Linak 1988) in operation during which inadequate temperature and mixing conditions in the combustion zone may lead to incomplete combustion.

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Hazardous waste sites often contain chlorinated solvents, including trichloroethylene (TCE). Combustion of TCE can lead to the formation of soot aerosols, whose extracts cause dioxin-like responses in cell cultures and medaka (*O. latipes*) embryos (Blankenship *et al*. 1994, Villalobos *et al*. 1996). Experiments on keratinocyte cultures showed that all of the hazardous material was associated with the aerosol and that little was found in the gas phase of the flames. Chemical analyses of the soot extracts indicated that chlorinated fulvalenes, among other chlorinated hydrocarbons, were major components of the mixture and that these may have been responsible for the toxic response. Using a fish model, Villalobos *et al*. (1996) examined whether materials with dioxin-like properties were present in the chemically complex TCE soot mixture and its fractions. The character of the effects caused by dioxin (e.g. cardiotoxicity and yolk sac oedema) in medaka (*Oryzias latipes*) embryos had been detected earlier (Wisk and Cooper 1990).

In the study by Villalobos *et al*. (1996), the whole dichloromethane (CH_2Cl_2) extract, a fraction of mixed polarity and a non-polar fraction were assayed. The whole extract (WE) and the two fractions described above, proved toxic or bioactive using a battery of bioassays. Biological potency was demonstrated *in vitro* both by measuring interference of compounds from the mixture with oestrogen receptor using rainbow trout (*Oncorhynchus mykiss*) liver cells, and by monitoring the mixture's binding affinity to the Ah receptor and further ability to convert it into its DNA binding form. The pattern of toxicity in medaka embryos was identical to that previously reported for dioxin. Chemical analyses documented the presence of at least 250 chlorinated incomplete combustion by-products in the whole soot extract. However, the obvious effector, the target compounds of the analysis, TCDD and TCDF, were not present at detectable (picomole) levels. Those results indicated that an array of toxic effects might arise from substances other than those targeted by conventional chemical analyses. They also suggested a need for bioassay-directed assessments of toxicity and/or biological potency in complex mixtures.

In this study, we exposed medaka embryos to the same TCE fractions as Villalobos *et al*. to examine cytochrome P450 1A and stress protein (hsp70 and hsp60) induction in early life stages of medaka (*O. latipes*). This provided an opportunity to evaluate the utility of using two biomarkers, EROD activity and stress protein expression, as indicators of response to the soot of chlorinated solvents. By exposing and examining medaka embryos, and continuing the effects into larval periods of development, we were able to compare the two biomarkers in a single system.

Materials and methods

Egg collection

Conditions and procedures for maintaining medaka and for collecting eggs have been described in DeKoven *et al*. (1992) and Marty *et al*. (1990). Within the first 4 h after fertilization, females were carefully netted and clusters of eggs removed from the ventral body wall using watchmaker's forceps. All clusters were placed in a Petri dish containing embryo-rearing medium (ERM, Kirchen and West 1976), chorionic filaments attaching eggs were broken, and individual eggs separated by carefully rolling egg clusters between moistened finger tips. After separation, embryonated eggs were placed in ERM and aerated vigorously. For the analysis of initial stage of development and embryo viability and growth, two independent observers used a dissecting microscope and observations were performed at least once a day for 8 days.

Exposures

For medaka embryo exposures, stock solutions of soot whole extract (WE) and fractions with different polarity, including fraction 1 (Fr1, non-polar compounds), fraction 2 (Fr2, containing primarily PAHs), fraction 3 (Fr3, intermediate polarity), and fraction 4 (Fr4, polar compounds) were dissolved in ERM (pH 7.2) using dimethylsulphoxide (DMSO) as vehicle solvent. Three different concentrations of incomplete combustion by-products (nominal) of soot WE were used: 2.7, 7.2, and 18 μg l^{-1}. For the four TCE fractions, one concentration (18 μg l^{-1}) was used. The nominal concentration of dioxin (2,3,7,8-TCDD) was 3 ng l^{-1}. The vehicle concentrations were restricted to 500 μl l^{-1} (0.05%), i.e. the concentration in which no embryonal toxicity was previously found (Villalobos *et al*. 1996). Controls consisted of embryos exposed to vehicle (C/Fr1, C/Fr2, C/Fr3, C/Fr4, C/WE) or ERM (C/ERM). To determine relevant concentrations of the soot extract and the fractions, range finding pilot assays were performed and the results used to determine conditions for the final test.

Fifty embryonated eggs (stage 10; Yamamoto 1975) were randomly distributed into individual 200 ml borosilicate glass vials containing 50 ml ERM. Each treatment consisted of two replicates. For the exposures, ERM was removed from the vials and replaced by 50 ml of the test solutions. Test vials were sealed with screw-type lids and Teflon tapes. They were kept at 25 ± 0.5 °C in a static, non-renewal system for 8 days, which is the time needed for control embryos to develop to stage 36. After this period, approximately 50 embryos were removed, rinsed in homogenizing buffer (50 mM Tris, containing 1mM EDTA and 0.25 M sucrose, pH 7.4), frozen immediately in liquid nitrogen, and stored at –80 °C until biomarker analyses. The remaining embryos were transferred into 50 ml ERM (control) solution and observed daily for viability. Dead embryos were removed. Embryos were allowed to hatch in clean ERM (1–4 days after ending the exposures), and newly hatched larvae were collected for EROD activity (approximately 30 larvae/treatment), hsp analyses (10 larvae/treatment), and immunohistochemical analyses (10 larvae/treatment).

Tissue preparations for immunohistochemistry

For cytochrome P450 1A (CYP1A) immunohistochemistry, 10 newly hatched larvae were frozen and transferred into a freeze-dryer (Tis-U-Dry freeze dryer, FTS Systems Inc., Stone Ridge, New York). The detailed method for freeze-drying has been described by Teh and Hinton (1993). After freeze-drying, larval samples were embedded in glycolmethacrylate (GMA, JB-4, Polysciences Inc., Warrington, Pennsylvania) and after polymerization, sections (2–4 μm) were cut using an LKB historange microtome and glass (Ralph) knives. With this method, tissues are not subjected to temperatures above 0–4 °C and dehydration is achieved without loss of enzyme activity. The method has proven satisfactory for immunohistochemical procedures as well (Teh and Hinton 1993).

Preparation of embryo and larval tissues for biochemical and immunochemical assay

For EROD activity, three sets of embryo and larval homogenates were prepared, pooling 10 embryos or larvae from the same treatment group. Pooled samples were homogenized in 100 μl homogenization buffer by hand on an ice bath using a tissue grinder and a Teflon pestle. The homogenates were centrifuged for 20 min at 9000×*g* (S9-fraction) at 4 °C. The total protein concentration in each supernatant was determined by a Bio-Rad DC Protein Assay. Samples were immediately frozen in liquid nitrogen, and stored at –80 °C until time of assay.

For hsp analysis, two to three embryos or three to four larvae were pooled for one sample, then homogenized by hand on ice in a glass homogenizer for 2 min with 50 μl of hypotonic solution containing 66 mM Tris buffer (Trizma Base, Sigma, pH 7.5), 1% Nonidet P-40 (Sigma), 1% SDS, and 0.2 mM phenylmethylsulphonylfluoride (Sigma). Homogenates were centrifuged at 7000×*g* for 30 min. at 4 °C. The total protein concentration of the samples was determined as for EROD assays. Sample buffer (Laemmli 1970) was immediately added to each sample at a 5:1 dilution, and proteins were denatured by heating to 90 °C for 2 min.

Enzyme histochemistry

Microscopic sections (2–4 μm thickness) were placed on electrostatically-charged glass slides and were air-dried. Monoclonal antibody (Mab 1-12-3p5) against scup cytochrome P450E (now CYP 1A1) (Kloepper *et al*. 1987) was used. Slides were stored at 4°C (max. 4 days) until immunohistochemistry was performed. Endogenous peroxidase activity was blocked by incubating the slides in 5% H_2O_2 (aqueous) for 5 min, followed by washing three times for 5 min with 0.01 M phosphate buffer with Tween (pH 7.4). Non-specific antibody binding was blocked by incubating slides with 0.5% normal horse serum for 20 min. The primary antibody was applied at a dilution of 1:500 at 4 °C overnight (18 h) in a humidified chamber. Biotinylated horse anti-mouse IgG was used as secondary antibody. Visualization was made by using a DAB Substrate Kit (Vector Laboratories, Burlingame, USA). The slides were counter-stained with haematoxylin-eosin and viewed.

Embryonal and larval EROD bioassay

Activity of hepatic 7-ethoxyresorufin O-deethylase (EROD) was measured fluorometrically (Cambridge Instruments 7620) according to the method of Burke and Mayer (1974), adapted for a microplate format. EROD assays were carried out on S9-fractions of eggs and larvae (see Tissue Preparations for Immunohistochemistry). Microsomes (600–800 µg protein/well) were incubated in 100 mM potassium phosphate buffer of pH 8, 2.5 µM ethoxyresorufin (Sigma Chemical Co.), and 0.5 mM NADPH (Sigma Chemical Co.) to a final volume of 200 µl. Fluorescence (excitation 530, emission 584) was recorded at 30 s intervals for 4 min at 25 °C.

Stress proteins

Samples containing 50 µg total protein were loaded on a 10 % polyacrylamide gel with a 5 % stacking gel using the buffer system described by Laemmli (1970). Each gel was run at 25 mA for approximately 1.5 h (Bio-Rad Mini-Protean System).

The gels were equilibrated in transfer buffer for 30 min, and proteins electroblotted onto Immobilon-P membrane (Millipore) (Towbin *et al.* 1979) at constant current (200 mA) for 90 min. A standard (Sigma, see below) was used and blotted proteins were stained with Ponceau solution to check for transfer efficiency. Membranes were blocked with 5 % skim milk in 20 mM Tris buffer and 0.4 M NaCl (pH 7.5) with 0.05 % Tween-20 for 30 min. The following primary antibodies were used as probes: polyclonal antibody, directed against hsp60 from *Synechococcus* sp. (cyanobacteria; Stressgen), dilution 1:1500; monoclonal, anti-hsp70 (MA3-001) antibody (Affinity Bioreagents), dilution 1:500. After examining the cross-reactivity of the primary antibodies with medaka heat shock proteins, each plot was probed with both anti-hsp60 and anti-hsp70. Blots were incubated for 90 min with primary anti-hsp60 antibody and then washed three times for 30 min in Tris-buffered saline solution containing 0.05 % Tween-20. Alkaline phosphatase-conjugated, secondary antibodies, anti-rabbit (Bio-Rad, 1:1000) was used to detect the hsp60 antibody probes. Membranes were washed again, incubated for 90 min with primary anti-hsp70 antibody and washed three times. Anti-rat IgG (Sigma, 1:10 000) was used as the secondary antibody to detect anti-hsp70. After washing, the substrates *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were used to visualize the bound antibodies. The developed blots were scanned on a densitometer (Bio-Rad GS-670).

One of the proteins contained in Sigma HMW protein standard cross-reacted with anti-hsp60, which was used as an internal standard to check for transfer efficiency. Additionally, gels were stained with Coomassie blue after transfer to ensure that complete protein transfer had occurred. Being aware of the semi-quantitative nature of western blotting, two or three samples from control treatments were incubated on each blot. The Western blots were collected and incubated and developed simultaneously to ensure that blot development conditions were identical. Fresh antibody solutions were used each time.

Statistical analysis

Differences between the treatment and the control groups were determined using one-way analysis of variance followed by Tukey HSD test. Differences were considered significant if $p < 0.05$. All statistical results were calculated in SPSS®.

Results

Mortality and condition of fish

During the exposure period, highest incidence of mortality (6 %) was observed in the WE group (WE 18). In ERM, Fr3 and WE 2.7 mortality was 2 %. Embryo mortality was not observed in C/Fr1, C/Fr2, C/Fr3, C/Fr4, Fr1, Fr2, Fr4, WE 7.2, or 2,3,7,8-TCDD. Apparent bacterial contamination was observed in some vials with the result that exposure solutions became cloudy. The resulting lack of test organisms did not allow us to obtain biomarker data for some of these treatments (larval EROD activity: C/Fr1, C/Fr2, C/Fr4, Fr2, WE 2.7, WE 18; embryonal hsp levels: C/Fr1, C/Fr2, Fr1, Fr2; larval hsp levels: C/Fr1, C/Fr2, Fr2, WE 18).

Embryonal and larval EROD activity

The effect of an 8-day exposure (of stage 10 medaka embryos) to the fractions or WE on EROD activity of medaka embryos and larvae is illustrated in figures 1 and 2

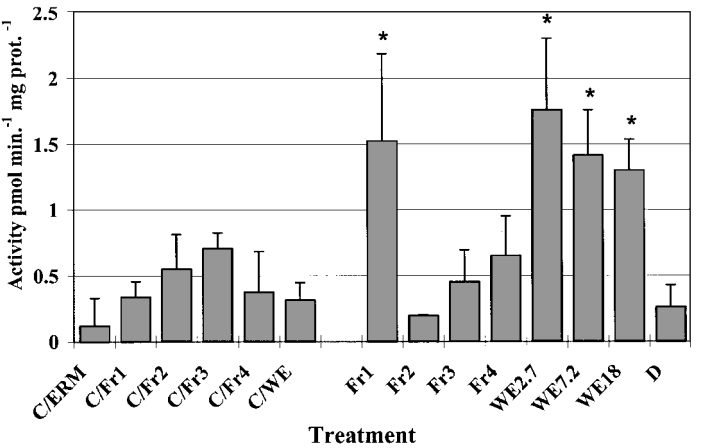

Figure 1. Embryonal EROD activity. Embryos were exposed beginning as stage 10 (on day of fertilization) and continuing for 8 days. Activity was determined as described in Materials and Methods and expressed as pmol min^{-1} mg^{-1} S9 protein. Each value represents mean of triplicate pools of 10 embryos each. * = $p < 0.05$ vs appropriate control. C/ERM—control using embryo rearing medium (ERM) only. C/Fr1—carrier vehicle (dimethyl sulphoxide and *n*-hexane) control for fraction 1 in ERM. C/Fr2—carrier vehicle (dimethyl sulphoxide and *n*-hexane/CH_2Cl_2) control for fraction 2 in ERM. C/Fr3—carrier vehicle (dimethyl sulphoxide and CH_2Cl_2) control for fraction 3 in ERM. C/Fr4—carrier vehicle (dimethyl sulphoxide and methanol) control for fraction 4 in ERM. C/WE—carrier vehicle (dimethyl sulphoxide) control for whole extract in ERM. Fr 1—dimethyl sulphoxide (500 µl l^{-1}) and *n*-hexane fraction (18 µg l^{-1}) in ERM. Fr 2- dimethyl sulphoxide and *n*-hexane/CH_2Cl_2 fraction (18 µg l^{-1}) in ERM. Fr 3—dimethyl sulphoxide and CH_2Cl_2 fraction (18 µg l^{-1}) in ERM. Fr 4—dimethyl sulphoxide and methanol fraction (18 µg l^{-1}) in ERM. WE 2.7—dimethyl sulphoxide and whole extract (2.7 µg l^{-1}) in ERM. WE 7.2—dimethyl sulphoxide and whole extract (7.2 µg l^{-1}) in ERM. WE 18—dimethyl sulphoxide and whole extract (18 µg l^{-1}) in ERM. D—2,3,7,8-TCDD in dimethyl sulphoxide (3 ng l^{-1}) in ERM.

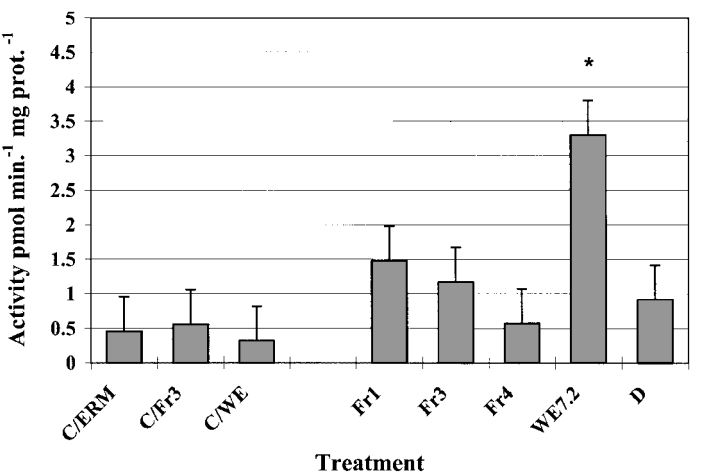

Figure 2. Larval EROD activity in medaka exposed as embryos from stage 10 and continuing for 8 days. Methods and other designations are identical to those in figure 1. Those treatments with apparent bacterial contamination were removed. Each value represents triplicate pools of 10 one-day-old hatchlings (larvae). *=$p < 0.05$ vs appropriate control.

respectively. EROD activity remained low in the embryonal control groups ranging from 0.12 (ERM only) to 0.55 pmol min^{-1} mg $prot.^{-1}$ (C/Fr3), with mean activity of 0.42 ± SD 0.12. Within the TCE-soot WE-exposed embryos, the EROD activity was highest in WE 2.7 (mean 1.76 pmol $min.^{-1}$ mg $prot.^{-1}$). The other WE-exposed groups, WE 7.2 and WE 18, also showed induced activity (1.41 and 1.30 pmol min^{-1} mg $prot.^{-1}$, respectively). The activities were significantly ($p < 0.05$) different from the control groups. Thus, the induction was 5.6× (WE2.7), 4.5× (WE7.2) and 4.1× (WE18), when compared with the respective control activity, showing a decreasing trend in EROD activity with increasing WE concentration. Within the groups exposed to the fractions, only fraction 1 (Fr1, non-polar compounds) showed significantly increased EROD activity (1.52 pmol min^{-1} mg $prot.^{-1}$; $p < 0.05$), which was similar to EROD activity in the WE groups. Activities in fraction 2 (Fr2), 3 (Fr3), and 4 (Fr4) were considerably lower and did not differ from the respective controls. Dioxin at a concentration of 3 ng l^{-1} did not induce EROD activity in embryos.

Larval EROD activity was significantly induced in one WE group, WE 7.2 (3.30 pmol min^{-1} mg $prot.^{-1}$). Larvae from other WE treatment groups (2.7, and 18) were not analysed due to mortality and bacterial contamination during the exposure experiments. The levels of EROD activity in embryo and larval control groups were not statistically different, but mean larval activity was 1.9 times higher in WE 7.2-treated larvae than in WE 7.2-exposed embryos (figure 2). EROD activity in larvae exposed as embryos to dioxin at a concentration of 3 ng l^{-1} was at control levels.

Localization of CYP 1A

Immunoreactive protein was localized in tissues and organs of larval medaka which were exposed as embryos to WE, dioxin or various TCE fractions. Photomicrographs of medaka exposed to WE 2.7 were selected for illustration of sites of localization (figures 3 and 4). Even at low magnification (figure 3), CYP 1A was seen in liver, gut, chondrocytes of cranium and tail, cornea and kidney. With higher magnification, increased detail was obtained (figure 4). Reactive protein was present in mucosal epithelial cells of kidney tubules, intestinal bulb and intestine. Liver and gall bladder were also positive and staining was apparent in hepatocytes and mucosal epithelium of the gall bladder, respectively. The resolution was not sufficient to determine if biliary ductular or ductal epithelium was positive. At higher magnification (figure 4), cells of the gas gland and epithelium of the swim bladder stained positively. Localization in controls was faint and was not seen in sites where positive staining was observed in WE 2.7-exposed larvae.

Stress proteins in embryos and larvae

In medaka embryos, no significant induction of hsp70 and hsp60 over respective control values was detected in any treatment group (figure 5). In control samples C/Fr3, hsp70 and hsp60 levels were significantly higher than in other control groups.

Figure 6 shows values for hsp60 and hsp70 for each of the control and exposure groups of medaka larvae. Differences in relative density between larval groups and corresponding groups of embryos are apparent (compare *y*-axes, figures 5 and 6). It

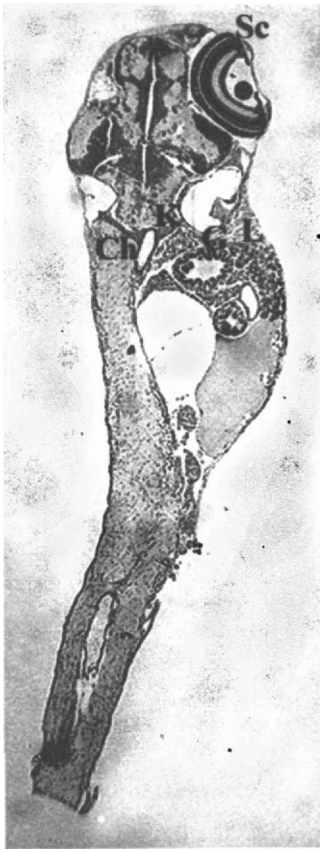

Figure 3. Section through freeze dried and glycolmethacrylate embedded medaka larva exposed for 8 days as an embryo to whole extract 2.7 μg l^{-1}. Sites of immunoreactive protein CYP 1A1 are shown. Enzyme localization methods are described in Materials and Methods. Labelling is in sclera of orbit (Sc), kidney (K), liver (L), chondrocytes (Ch) of cranium and spinal cord and gut (G). Bar = 1 mm.

was hsp70 that comprised the greatest proportion of the response in larvae. Larval hsp70 was significantly induced in the control for fraction 3 (C/Fr3), as seen in embryo samples, but not in other control groups. In larvae exposed as embryos to TCE soot extract (WE) at concentrations of 2.7 and 7.2 μg l^{-1} and to TCDD at 3 ng l^{-1} concentration, hsp70 and hsp60 expression was significantly increased over the respective control group (C/WE). Samples from the WE 18 treatment were not available due to increased mortality (see above) during the exposure experiment. Although not statistically significant, the mean value for the Fr4 (polar compounds) larval group was increased over the corresponding embryo group and over all control groups except C/Fr3 (figures 5 and 6).

Discussion

This study was a sequel to previous investigations (Blankenship *et al.* 1994, Villalobos, *et al.* 1996) which had shown that there were dioxin-like properties of a trichloroethylene combustion-generated aerosol despite the failure to detect dioxin

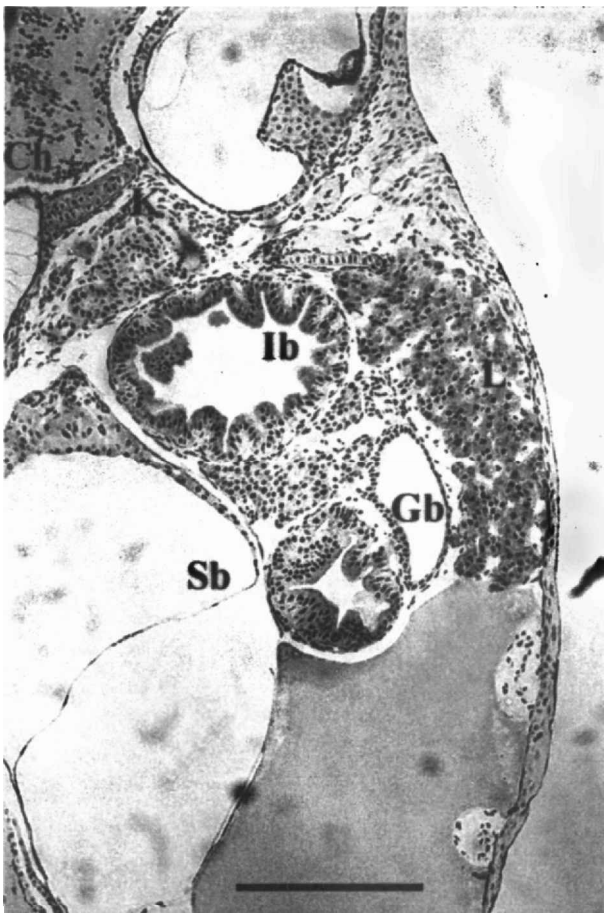

Figure 4. Higher magnification of section shown in figure 3. Immunoreactive protein is in chondrocytes of cranial cartilage (Ch), tubular epithelial cells of kidney (K), mucosal epithelium of intestinal bulb (Ib), hepatocytes of liver (L), mucosal cells of gall bladder (Gb), epithelial cells lining swim bladder (Sb) and in mucosal epithelial cells of intestine. Bar = 250 μm.

at picomole levels of sensitivity. The present study investigated embryonic and larval responses following embryo exposure to the same extracts and fractions that were originally investigated in Villalobos *et al.* (1996). In that previous study, the whole extract concentration in which 50 % of larvae showed signs of abnormality was 7.2 $\mu g\ l^{-1}$, while at 2.7 $\mu g\ l^{-1}$, no effect was observed. Of the whole extract fractions, only fraction 3 (Fr3, intermediate polarity compounds) at a concentration of 18 $\mu g\ l^{-1}$ (which was also used in our study) was associated with statistically significant decreases in normal larval development.

The design of the present study involved the same duration of exposure, with initiation of exposure at stage 10 (day 1, multi-cell stage) and continuing for 8 days. Upon termination of exposure, eggs were rinsed, placed in clean ERM and maintained under standard culture conditions until hatch. Only normal appearing, viable larvae were used. This means that larvae included in the present study were restricted to individuals whose responses could be detected only by molecular, biochemical, or microscopic analysis. Embryos from the whole extract exposure

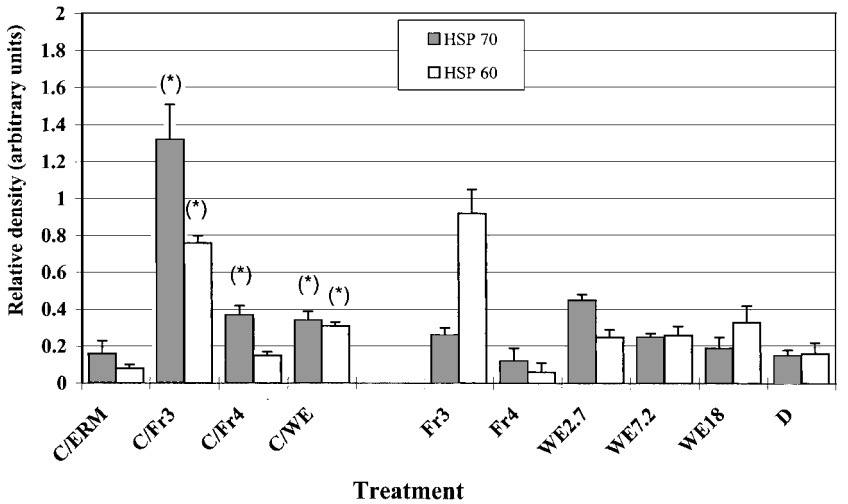

Figure 5. Histogram of relative densities for hsp60 and hsp70 in medaka embryos. Shown are mean densities of protein bands detected by western blotting ± standard errors ($n = 3$–5). Embryos were exposed beginning as stage 10 on day of fertilization and continuing for 8 days. (*) significantly different from C/ERM ($p < 0.05$) C/ERMcontrol using embryo rearing medium in ERM. C/Fr3—carrier vehicle (dimethyl sulphoxide and CH_2Cl_2) control for fraction 3 in ERM. C/Fr4—carrier vehicle (dimethyl sulphoxide and methanol) control for fraction 4 in ERM. C/WE—carrier vehicle (dimethyl sulphoxide) control for whole extract in ERM. Fr 3—dimethyl sulphoxide and CH_2Cl_2 fraction (18 μg l^{-1}) in ERM. Fr 4—dimethyl sulphoxide and methanol fraction (18 μg l^{-1}) in ERM. WE 2.7—dimethyl sulphoxide and whole extract (2.7 μg l^{-1}) in ERM. WE 7.2—dimethyl sulphoxide and whole extract (7.2 μg l^{-1}) in ERM. WE 18—dimethyl sulphoxide and whole extract (18 μg l^{-1}) in ERM. D—2,3,7,8-TCDD in dimethyl sulphoxide (3 ng l^{-1}) in ERM.

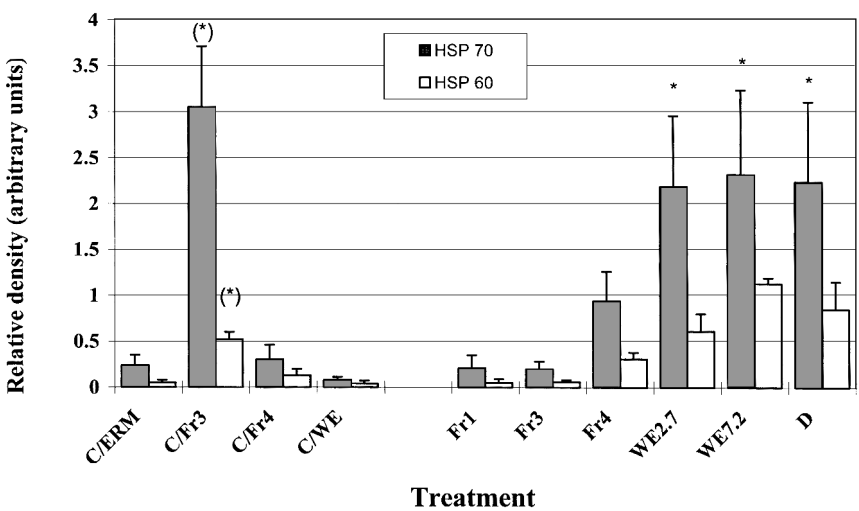

Figure 6. Histogram of relative densities for hsp60 and hsp70 in medaka larvae exposed as embryos from stage 10 and continuing for 8 days. Larvae were collected within on day of hatch and within 4 days following termination of exposure. Shown are mean densities of protein bands detected by western blotting ± standard errors ($n = 3$–4). *significantly different from respective control ($p < 0.05$). (*) significantly different from C/ERM ($p < 0.05$). All designations are as in figure 5 above.

groups and the group exposed to fraction 1 showed induction of EROD activity. Despite the fact that fraction 3 proved embryotoxic in the 1996 study, this fraction was not associated with induction of embryonal EROD activity. In larvae, correlation between EROD induction and embryotoxicity was seen for embryos exposed to whole extract at a concentration of 7.2 μg l^{-1}. Mean EROD activity was double that of control values in embryos exposed to fraction 1 and to fraction 3, but this increase was not statistically significant. These results imply that although exposure has occurred, time and perhaps development are needed before the response can be observed. Increasing the concentration of whole extract appeared to have an inhibitory effect upon embryonal EROD activity. It is possible that cellular toxicity, which would be expected to increase with increasing concentration of whole extract, could have led to death of cells reducing the organism-level response. Detailed morphologic investigations are underway to address this question.

We were somewhat surprised when dioxin failed to induce EROD activity. Previous studies in this laboratory (Anderson *et al.* 1996) used molar TCDD concentrations from 10^{-7} to 10^{-13} and observed response in studies with the oestrogen receptor and the nuclear response element. Recent studies with dioxin involving seven freshwater fish species during early life-stage development used higher concentrations, 9–285 ng l^{-1} (Elonen *et al.* 1998). We are repeating elements of the dioxin study with a broader range of concentrations. However, as stated below, there was an hsp70 response in larval medaka exposed to this compound as embryos.

The immunohistochemical localization of the CYP1A-reactive protein showed that the liver was a major site for this important component of the mixed function oxidase system. If the areal analysis is representative, post-mitochondrial supernatant derived EROD values likely reflect activity in this organ. This implies that the liver should be a site of investigation for toxicity produced by increasing concentrations of the whole extract. Our localization studies for stress proteins in medaka are continuing and we will attempt to determine whether the stress protein response and the EROD activity are related to the same organs and tissues.

With the exception of samples from the control/Fr3 treatment group, significant hsp70 or hsp60 induction did not occur in exposure groups until the larval stages. This implies that time and development were necessary for a response to be seen. Correlation was good between those WE concentrations which produced embryotoxicity and those that caused significant hsp70 and, in some cases, hsp60 induction. In addition, induction of both stress proteins following exposure to a concentration of WE (2.7 μg l^{-1}) that failed to induce embryotoxicity indicates the sensitive nature of the response. The induction of EROD activity in embryos and the absence of such induction for hsp until larval samplings suggest that either cellular damage leading to hsp induction (e.g. aberrant protein formation) takes time to develop, or that the ability for hsp to be induced is a property of later developmental stages. Exposure to dioxin did not result in induction of EROD activity, but it was associated with statistically significant hsp70 and hsp60 induction. This finding suggests that, for dioxin, the stress protein response is the more sensitive of the two biomarkers. It is known that stress proteins, in particular members of the hsp90, but also hsp70 and hsp60 proteins, are involved in aryl hydrocarbon receptor (Ah-receptor) activation and function (Whitelaw *et al.* 1993, Blankenship and Matsumura 1997, Pratt and Toft 1997, Caruso *et al.* 1999). The Ah-receptor is a ligand-activated transcription factor that mediates the effects of agonists like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. We know

of no study that investigated the effect of dioxin on cellular hsp70 and hsp60 expression directly. However, LaPres *et al.* (2000) were able to show that TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) results in an increase of Ah-receptor in the cytosol, and that the associated hsp and ARA9 molecules stabilized the receptor under heat stress. It appears reasonable to assume that, even in the absence of cellular damage, the concentration of complex-forming proteins would increase in response to dioxin along with an increase of receptor concentration.

We are lacking an obvious explanation for elevated hsp60 and hsp70 levels in C/Fr3 embryos and larvae. This treatment was strongly affected by 'apparent bacterial contamination'. The term 'apparent bacterial contamination' was used because no bacterial isolation and identification was performed on contaminated vials with cloudy fluid. Several studies suggest that bacterial infection can induce both stress proteins (Forsyth *et al.* 1997, Belles *et al.* 1999, Gregorc and Bowen 1999), potentially as part of the affected organism's immune response (Beimnet *et al.* 1996, Stulik *et al.* 1999). Other studies did not demonstrate increased expression of hsp as a consequence of bacterial infection (e.g. Mantis *et al.* 1996). It is possible that contaminating microorganisms were able to penetrate the chorion and infect the embryos. This infection would subsequently affect the larvae as well, which could explain elevated stress protein levels in both embryos and larvae from this treatment. The intensity of the response to contamination in the C/Fr3 group suggests that—had the contaminant been present in other treatments—enhanced hsp expression would have occurred in both embryos and larvae. This is not the case in the whole soot extract- and dioxin-treated groups. In addition, no cloudiness of the test solution was observed in the latter treatments. It is therefore likely that the induction of hsp60 and hsp70 detected in larvae after embryonal exposure to the whole extract solutions and dioxin is independent of bacterial infection. The contamination in C/Fr3 did not affect EROD activity, which confirms the higher specificity of this biomarker in comparison with stress proteins. Stress proteins can be sensitive indicators, but they are involved in a multitude of cellular functions and therefore rather unspecific. This underlines the importance of analysing several biomarkers in the same system, i.e. looking at it from 'different angles', to facilitate interpretation of results.

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