Antioxidant Responses and NRF2 in Synergistic Developmental Toxicity of PAHs in Zebrafish

Alicia R. Timme-Laragy,*,1 Lindsey A. Van Tiem,* Elwood A. Linney,*,† and Richard T. Di Giulio*,2

*Integrated Toxicology and Environmental Health Program, Nicholas School of the Environment, Duke University, Durham, NC 27708; and †Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710

Early piscine life stages are sensitive to polycyclic aromatic hydrocarbon (PAH) exposure, which can cause pericardial effusion and craniofacial malformations. We previously reported that certain combinations of PAHs cause synergistic developmental toxicity, as observed with coexposure to the aryl hydrocarbon receptor agonist β-naphthoflavone (BNF) and cytochrome P4501A inhibitor α-naphthoflavone (ANF). Herein, we hypothesized that oxidative stress is a component of this toxicity. We examined induction of antioxidant genes in zebrafish embryos (*Danio rerio*) exposed to BNF or ANF individually, a BNF + ANF combination, and a prooxidant positive control, *tert*-butylhydroperoxide (tBOOH). We measured total glutathione (GSH) and attempted to modulate deformities using the GSH synthesis inhibitor L-buthionine (*S,R*)-sulfoximine (BSO) and increase GSH pools with N-acetyl cysteine (NAC). In addition, we used a morpholino to knockdown expression of the antioxidant response element transcription factor NRF2 to determine if this would alter gene expression or increase deformity severity. BNF + ANF coexposure significantly increased expressions of superoxide dismutase 1 and 2, glutathione peroxidase 1, pi class glutathione-*s*-transferase, and glutamate cysteine-ligase to a greater extent than tBOOH, BNF, or ANF alone. BSO pretreatment decreased some GSH levels, but did not worsen deformities, nor did NAC diminish toxicity. Knockdown of NRF2 increased mortality following tBOOH challenge, prevented significant upregulation of antioxidant genes following both tBOOH and BNF + ANF exposures, and exacerbated BNF + ANF–related deformities. Collectively, these findings demonstrate that antioxidant responses are a component of PAH synergistic developmental toxicity and that NRF2 is protective against prooxidant and PAH challenges during development.

Key Words: PAH; NRF2; redox; ROS; embryonic development; glutathione.

Many pollutants cause perturbations in cellular redox and can generate reactive oxygen species (ROS) (Livingstone, 2001; Valavanidis *et al.*, 2006). While ROS are produced endogenously via respiration and oxygenating enzymes (Droge, 2003), oxidative stress can result when there is "a disruption of redox signaling and control" (Jones, 2006b). This is a mechanism of toxicity of numerous xenobiotics, and contributes to maladies ranging from chemical carcinogenesis to neurodegenerative diseases (Droge, 2003; Valavanidis *et al.*, 2006). Embryonic development may be especially sensitive to oxidative stress, as even a 15%–20% increase in ROS can tip progenitor cells into premature cell cycle arrest and differentiation (Li *et al.*, 2007; Smith *et al.*, 2000). In mammals, *in utero* oxidative stress can alter gene expression persisting through adulthood, affecting DNA methylation, and increasing cancer susceptibility (Wan and Winn, 2006). Thus, toxicant exposures that cause oxidative stress during embryonic development are a significant health risk.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants produced by the incomplete combustion of organic compounds, and concentrations are increasing with usage of fossil fuels (Van Metre and Mahler, 2005). Complex mixtures of PAHs commonly include four- and five-ring structures that can have variable interactions with the aryl hydrocarbon receptor (AHR) pathway. The AHR is a member of the basic helix-loop-helix per-ARNT-Sim transcription factor family (Gu *et al.*, 2000); ligand binding to the AHR upregulates several detoxifying enzymes, including cytochrome P4501A (CYP1A), important in metabolism of PAHs. While some PAHs are excellent agonists for the AHR, others can inhibit the activity of CYP1A. Coexposures to PAHs with these characteristics cause synergistic embryotoxicity characterized by craniofacial malformations and cardiovascular deformities including pericardial effusion (Billiard *et al.*, 2006; Hodson *et al.*, 2007; Wassenberg and Di Giulio, 2004).

PAHs can generate ROS via production of PAH metabolites that include redox-cycling quinones and radical cations, the latter being associated with DNA damage (Cavalieri and Rogan, 1992; Melius, 1984; Miranda *et al.*, 2006). Also, PAHs can damage mitochondrial DNA and perturb mitochondrial function, which may enhance oxidative stress in these organelles (Li *et al.*, 2003; Zhu *et al.*, 1995). Induction of CYP450s via the AHR by compounds such as chlorinated dioxins and PCBs can lead to ROS generation and oxidative

[1] Present address: Biology Department, Woods Hole Oceanographic Institute, Woods Hole, MA 02543
[2] To whom correspondence should be addressed at Duke University, Research Drive, Levine Science Research Center, Room A346, Durham, NC 27708-0328. Fax: (919)-668-1799. E-mail: richd@duke.edu.

damage (Nebert *et al.*, 2000; Schlezinger and Stegeman, 2001), associated with the recalcitrance of these compounds to metabolism. Numerous PAHs also induce CYP450s via the AHR, but this induction has not been thought to generate ROS *per se*, due to the suitability of PAHs as CYP substrates. However, ROS generation may be enhanced in coexposures to PAHs which include both AHR agonists and CYP inhibitors, exposures which give rise to synergistic embryotoxicity (Billiard *et al.*, 2006; Wassenberg and Di Giulio, 2004).

Several studies have observed indications of oxidative stress in fish exposed to PAHs. Rainbow trout larvae exposed to the alkylated-PAH retene had reduced glutathione (GSH): glutathione disulfide ratios and whole-body vitamin E concentrations (Bauder *et al.*, 2005). Killifish from a PAH-contaminated Superfund site had elevated total GSH and mitochondrial lipid peroxidation (Bacanskas *et al.*, 2004). Laboratory-raised progeny from this site was better equipped than reference site F1s to withstand oxidative stress, with increased survival times to *tert*-butylhydroperoxide (tBOOH), total GSH, MnSOD protein, and GSH reductase activity (Meyer *et al.*, 2003). Altered antioxidant defenses, an indication of oxidative stress, appear to be acting in response to PAH exposure.

Many antioxidant defenses respond to the antioxidant response element (ARE) transcription factor, NF-E2 p45–related factor 2 (NRF2), a member of the cap'n'collar basic region-leucine zipper transcription factors. Under unstressed conditions, NRF2 is constitutively bound to Kelch-like ECH–associated protein 1 (KEAP1) in the cytoplasm and targeted for degradation (Nguyen *et al.*, 2003a). Upon ROS detected by KEAP1 or by redox-responsive signaling pathways (Cullinan and Diehl, 2006; Cullinan *et al.*, 2003; Huang *et al.*, 2000; Kong *et al.*, 2001; Zipper and Mulcahy, 2003), NRF2 translocates to the nucleus, associates with small MAF proteins, and binds to AREs in the promoter regions of a set of cytoprotective genes (Kobayashi *et al.*, 2006). This pathway is conserved in zebrafish (Kobayashi *et al.*, 2002; Li *et al.*, 2008), and NRF2 has been shown to be upregulated by AHR activation in mice (Miao *et al.*, 2005).

We hypothesized that oxidative stress is a component of the developmental toxicity of PAH mixtures. Zebrafish embryos were exposed to a combination of two oxygen-substituted model PAHs shown to result in synergistic toxicity, the AHR agonist β-naphthoflavone (BNF), and CYP1A inhibitor α-naphthoflavone (ANF) (Billiard *et al.*, 2006). We examined expression of several antioxidant genes, total GSH, and whether redox state could modulate pericardial effusion. We also knocked down NRF2 and asked whether this exacerbated deformities. Our results support oxidative stress as a component of the synergistic developmental toxicity of PAH mixtures and identify a protective role for NRF2 in embryonic oxidative stress. However, the precise role of oxidative stress in cardiovascular deformities due to BNF + ANF coexposures remains unclear.

MATERIALS AND METHODS

Chemicals. The chemicals ANF, BNF, N-acetyl cysteine (NAC), L-buthionine (*S,R*)-sulfoximine (BSO), GSH, naphthalene dicarboxaldehyde (NDA), n-ethylmorpholine, triscarboxyethylphosphine, and tBOOH were obtained from Sigma Aldrich (St, MO). Dimethyl sulfoxide (DMSO), potassium hydroxide, and sodium hydroxide were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Sulfosalicyclic acid was obtained from Ricca Chemical Company (Arlington, TX). ANF and BNF were dissolved in DMSO and kept protected from light at − 20°C and vortexed prior to use. NAC and BSO were dissolved in 30% Danieau water, and NAC was pH adjusted to 7.0 and stored at − 20°C; BSO was stored at 4°C.

Animals. Zebrafish embryos were collected within 1 h following mating of AB strain adults (From the laboratory of Dr Elwood Linney at Duke University and the Zebrafish International Research Consortium in Eugene, OR) maintained at 28°C on a 14-h light, 10-h dark light cycle. Adults were fed with TetraMin flake food and brine shrimp. Embryos were maintained in 30% Danieau water (Nasevicius and Ekker, 2000) under the same temperature and photoperiod conditions.

Exposure for gene expression experiments. At 24 hours post fertilization (hpf), embryos were dosed in replicate pools of five embryos, with three replicates per treatment, in 20-ml glass vials containing 7.5 ml 30% Danieau water. Final DMSO concentration was 0.03%. Coexposure experiments consisted of exposures to DMSO, 1 μg/l BNF alone, 100 μg/l ANF alone, and 1 μg/l BNF + 100 μg/l ANF in combination, sampled for RNA at 48 or 96 hpf. Coexposure experiments were repeated at least four times, and each data point represents an *n* of 12 pools of five embryos. The dose-response analysis for BNF was sampled at 48 hpf and included concentrations of 0, 1, 10, 50, and 100 μg/l; the concentration of 50 μg/l caused a slight increase in pericardial effusion, while 100 μg/l embryos appeared similar to those in the 1 μg/l BNF + 100 μg/l ANF coexposures at 96 hpf (Billiard *et al.*, 2006). Dose-response experiments were repeated at least twice, and each data point represents an *n* of at least six pools of five embryos. Embryos were left in original dosing solutions for the duration of the experiments. The model prooxidant tBOOH was used as a positive control. Three pools of five embryos were dosed at 24 hpf in glass scintillation vials in 7.5 ml of 30% Danieau water containing either 0 or 500μM tBOOH; this exposure was repeated 3 h prior to fixation at 48 hpf in order to run in parallel to tBOOH survival experiments and allow sufficient time for a transcription response after the second dose. No death or deformities were observed at this time point. Before sampling, embryos were rinsed with 30% Danieau water and manually dechorionated where necessary, then fixed in RNA later (Ambion, Foster City, CA), and stored at − 80°C.

Total RNA extraction and reverse transcription. RNA extractions were carried out according to the RNA easy MicroKit Protocol (Qiagen, Valencia, CA). RNA quantity and quality were analyzed spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). The Omniscript complementary DNA (cDNA) synthesis kit for Reverse Transcription (Qiagen) was used according to the manufacturer's instructions using 500 ng of RNA, random hexamers, and RNAse inhibitor and carried out in a thermocycler for 1 h at 37°C. Resulting cDNA was diluted to a working concentration of 2 ng/μl.

Quantitative real-time PCR. β-Actin primers were designed using Light Cycler Probe Design software (Roche, Indianapolis, IN). Primers for gstp1, gstp2, and gclc were designed using PrimerQuest software (Integrated DNA Technologies, Inc., www.idtdna.com). Due to high sequence similarity between gstp1 and gstp2 (90.4% sequence similarity), conserved regulatory elements, and inconsistent results with previously published primers (Suzuki *et al.*, 2005), we designed primers that would amplify both of these homologs at once, henceforth referred to as gstp. Sod2, Sod1, and gpx1 primers were published previously (Malek *et al.*, 2004). Primer efficiencies were also tested to ensure the housekeeping and target genes amplified at the same rate. The sequences employed are listed in Table 1.

TABLE 1
Primer and GenBank Accession Numbers for QRT-PCR

Gene	GenBank ID	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-Actin	AF057040	ACATCCGTAAGGACCTG	GGTCGTTCGTTTGAATCTC
Sod1	Y12236	CGCATGTTCCCAGACATCTA	GAGCGGAAGATTGAGGATTG
Sod2	AW07696	CTAGCCCGCTGACATTACATC	TTGCCCACATAGAAATGCAC
Gpx1	AW232474	AGATGTCATTCCTGCACACG	AAGGAGAAGCTTCCTCAGCC
Gstp	NM_131734; NM_199277	TCTGGACTCTTTCCCGTCTCTCAA	ATTCACTGTTGCCGTTGCCGT
Gclc	NM_199277	AACCGACACCCAAAGATTCAGCACT	CCATCATCCTCTGGAAACACCTCC

A 25 μl reaction contained 200nM of each primer, 12.5 μl 2× Sybrgreen Lightcycler Master Mix (Applied Biosystems, Foster City, CA), 9.5 μl dH$_2$O, and 4 ng cDNA template. quantitative real-time PCR (QRT-PCR) was carried out on an ABI 7300 quantitative real-time PCR machine under the following thermal cycle: 10 min at 95°C, followed by 40 replicates of 15 s at 95°C, then 1 min at 60°C, followed by a dissociation curve. All samples were run in duplicate.

QRT-PCR data were analyzed using the ABI PRISM 7000 Sequence Detection System, Version 1.1 (Applied Biosystems). The comparative C$_T$ method (Livak and Schmittgen, 2001) was used to determine average fold induction of messenger RNA (mRNA) by comparing the C$_T$ of the target gene to that of the reference gene (β-actin). Reference gene expression was not altered by treatment. Each technical replicate was averaged prior to data analysis. The fold change obtained for each biological replicate pool of five embryos was averaged for treatments; 6–15 replicates were used to obtain final induction averages and standard error per treatment per time point.

GSH quantification. Total GSH (GSH + glutathione disulfide [GSSG]) levels were quantified in pools of 25 embryos at 72 hpf that had been exposed
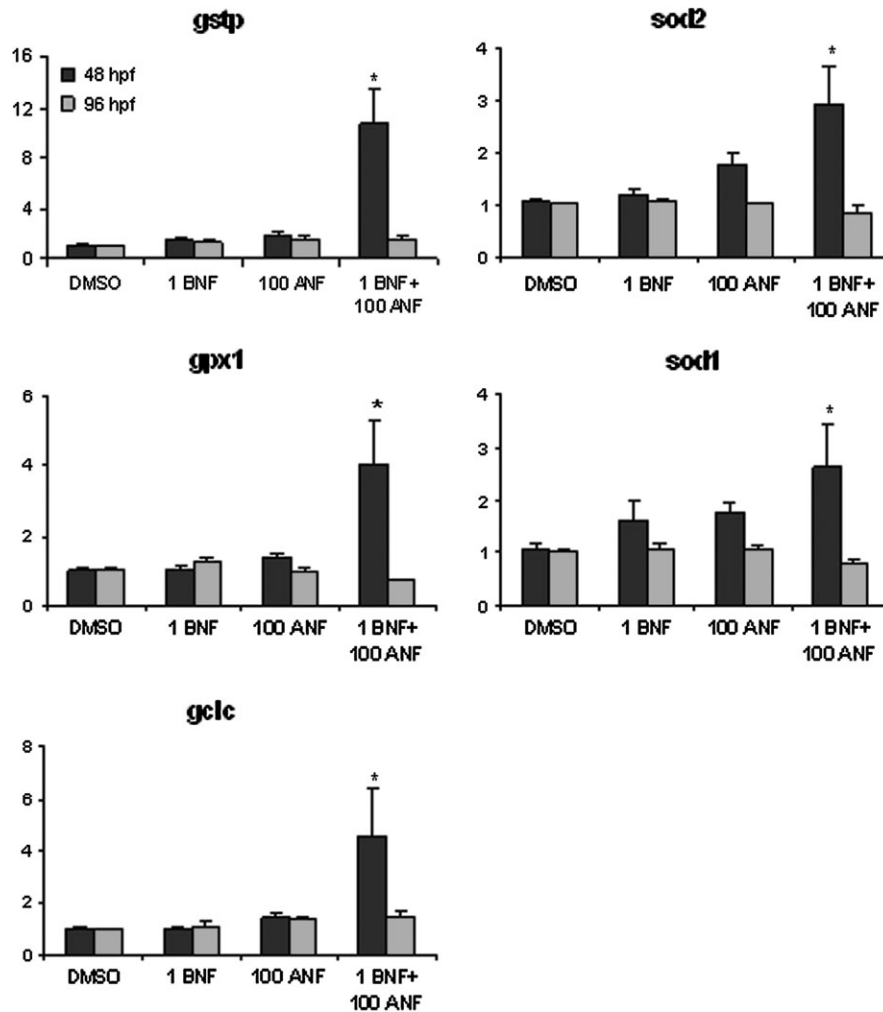

FIG. 1. Increase in expression at 48 but not 96 hpf of antioxidant genes gclc, gstp, gpx1, sod1, and sod2 in zebrafish embryos coexposed to BNF and ANF, but not either compound alone, compared to DMSO controls. All exposures began at 24 hpf. *$p \leq 0.003$. Data are presented as mean + SEM, $n \geq 12$.

to DMSO, 1 μg/l BNF, 100 μg/l ANF, or BNF + ANF from 48 to 72 hpf and included replicates of these doses that had been pretreated at 24 hpf with either 100μM NAC or 1000μM BSO. Embryos were washed with Danieau water and snap frozen in liquid nitrogen and stored at − 80°C until analysis. We used a plate reader assay based on the reaction of NDA with GSH or γ-GC to form highly fluorescent cyclized products to quantify total GSH (White *et al*., 2003). Fluorescence was read on a plate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) at an excitation of 470 nm and emission of 520 nm, quantified based on a linear standard curve and corrected for protein content. Protein was measured using the BioRad Protein microtiter plate assay with BioRad Protein Assay dye reagent concentrate (Hercules, CA), bovine serum albumin standards, and absorbance at 595 nm.

Redox modulation and deformity experiments. These experiments tested whether pretreatment with a GSH-modulating chemical, NAC or BSO, would alter severity of pericardial effusion resulting from BNF + ANF coexposures. Embryos were pretreated from 24 to 48 hpf and supplemented daily with BSO or NAC during a static 1 μg/l BNF + 100 μg/l ANF coexposure from 48 to 120 hpf. While the PAH exposure in these experiments is 24 h later than in the gene expression experiments, with respect to deformities, there is no difference between whether the embryos are dosed at 24 or 48 hpf (Timme-Laragy, unpublished results). Dosing was conducted in triplicate 20 ml glass scintillation vials containing pools of five embryos in a total volume of 7.5 ml 30% Danieau water, with a final DMSO concentration of 0.03% in all groups. At 120 hpf, embryos were washed with Danieau water, anesthetized in MS-222, mounted in 3% methylcellulose, and imaged in a left lateral orientation under ×50 magnification (Zeiss Axioskop, Thornwood, NY.). While other deformities including craniofacial malformations such as truncation of Meckel's cartilage are also observed and can be measured, we have found that pericardial effusion is a robust and corollary indicator of the severity of all related deformities (Billiard *et al*., 2006). Pericardial effusion area was quantified using IP Lab software (Scanalytics Inc., Fairfax, VA) and normalized to control pericardial area as described previously (Billiard *et al*., 2006). BSO experiments also included nontoxic BNF + ANF combination with a lower dose of ANF (50 instead of 100 μg/l) to determine whether there would be exacerbation of deformities; these were analyzed at 72 and 120 hpf.

Embryo morpholino injection. We used a morpholino (Gene Tools, Philomath, OR) that had been designed previously to block initiation of translation of zebrafish NRF2 mRNA, (5′-CATTTCAATCTCCATCATGTCT-CAG-3′) (Kobayashi *et al*., 2002). Gene Tools' standard control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used as the control morpholino (CO-MO) in experiments. Morpholinos were diluted to working stocks of 0.1mM in 30% Danieau's solution for injection (Nasevicius and Ekker, 2000). Injection working stocks were stored at 4°C and heated for 5 min at 65°C prior to use. Both morpholinos were tagged with a 3′-end carboxyfluorescein modification in order to monitor injection success.

Newly fertilized embryos at the one- to four-cell stage (up to approximately 1 hpf) were injected with approximately 3 nl of morpholino using a Narishige IM300 Microinjector (Tokyo, Japan). Only healthy embryos exhibiting strong, uniform fluorescence at 24 hpf were subsequently used in experiments.

Prooxidant survival exposures. For tBOOH survival experiments, embryos were sorted into individual wells in a 96-well plate at 24 hpf in 200 μl of 30% Danieau water. Noninjected, control morpholino–injected, and NRF2 morpholino–injected embryos were exposed to concentrations of 0, 0.1, 0.5, 0.75, 1, and 2mM tBOOH at 48 and 72 hpf. Cumulative mortality was analyzed at 96 hpf.

Statistical analysis. Data were analyzed with Statview for Windows (version 5.0.1; SAS Institute, Cary, NC). ANOVA was used to determine significant factors. When ANOVA yielded significance ($p < 0.05$), Fisher's protected least-significant differences was used as a *post hoc* test. Data are presented as mean ± SEM, and *n* defined as number of pools of five or 25 embryos. LC_{50} and 95% confidence intervals for tBOOH exposures were calculated using probit analysis in SPSS software (SPSS 15.0, SPPS Inc, Chicago, IL).

TABLE 2
Fold Changes of mRNA at 48 hpf by tBOOH (0.5mM) Compared to Induction by the BNF + ANF (1 + 100 μg/l) Coexposure

Gene	tBOOH	BNF + ANF
gclc	**2.8 ± 0.5**	**4.6 ± 1.8**
gstp	**6.7 ± 1.3**	**10.7 ± 2.8**
gpx1	**3.2 ± 0.6**	**4.1 ± 1.3**
sod2	1.6 ± 0.3	**2.9 ± 0.7**
sod1	1.4 ± 0.3	**2.6 ± 0.8**

Bold text indicates significant inductions compared to the respective vehicle controls ($p < 0.05$). $n \geq 12$ pools of five embryos.

RESULTS

Upregulation of Antioxidant Genes

We examined induction of several antioxidant genes by quantitative real-time PCR, both prior to onset of deformities at 48 hpf, and after deformity development at 96 hpf, in zebrafish embryos exposed to a dose of BNF + ANF that results in synergistic developmental toxicity. We observed a significant fold-change induction ($p \leq 0.003$) in the BNF + ANF coexposure groups compared to vehicle controls for gclc, gstp, gpx1, sod1, and sod2 at 48 hpf but not at 96 hpf (Fig. 1). At 48 hpf, gstp exhibited the highest induction of 10.7-fold, followed by gclc at 4.6-fold; both of these inductions were synergistic (two-factor ANOVA interaction terms 0.02 and 0.03). The next highest response was a 4.1-fold increase of gpx1. Sod2 and sod1 were the least responsive, yet still induced 2.9- and 2.6-fold, respectively. Individual doses of BNF (1 μg/l) and ANF (100 μg/l) did not result in a significant induction of any of
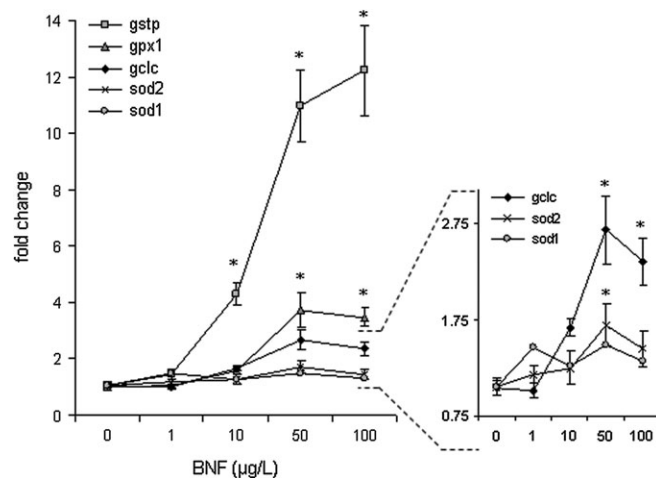

FIG. 2. The antioxidant genes gstp, gpx1, gclc, and sod2 are induced in a dose-dependent manner following 24-h exposure to BNF in 48 hpf zebrafish embryos. The inductions of gclc, sod1, and sod2 have been additionally plotted on an appropriate viewing scale (right). Following significant ANOVAs ($p \leq 0.03$), Fisher's protected least-significant differences significance above control is noted by (*$p \leq 0.04$). Data are presented as mean ± SEM where $n \geq 6$.
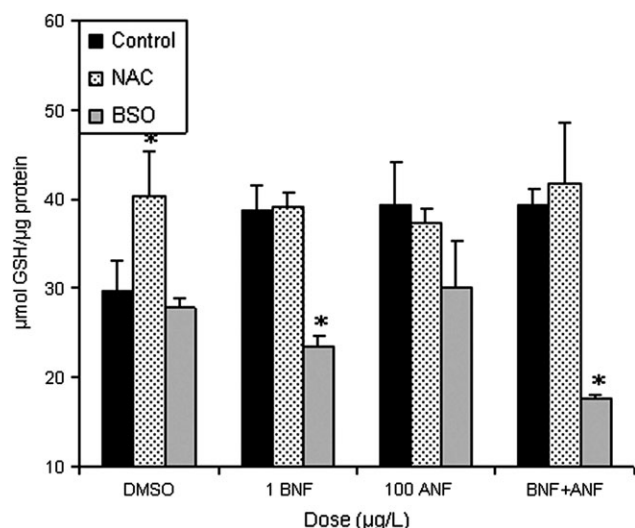
FIG. 3. Total GSH quantification at 72 hpf, with and without 100µM NAC and 1mM BSO. NAC and BSO treatments spanned 24–72 hpf and PAH and vehicle control treatments spanned 48–72 hpf. A three-factor ANOVA for pretreatment, BNF, and ANF showed a significant effect of NAC ($p = 0.03$) and BSO ($p < 0.0001$). *indicates a significant difference ($p \leq 0.05$) of either NAC or BSO pretreatment on GSH levels compared to no pretreatment controls within an exposure group; mean + SEM; $n = 3$ pools of 25 embryos.

these genes. No significant differences in antioxidant gene induction were observed after deformity onset at the 96 hpf time point, unlike the constant activation of the AHR, which continues to upregulate cyp1a, cyp1b1, cyp1c1, and ahrr2 at 96 hpf time points (Timme-Laragy *et al.*, 2007).

Significant inductions of gstp, gpx1, and gclc were also observed at 48 hpf in embryos exposed to a sublethal dose of tBOOH (0.5mM; no mortality at this time point), and induction levels were similar to or less than those observed in the 48 hpf BNF + ANF embryos (Table 2). The induction of antioxidant genes is indicative of redox signaling perturbations, ROS, and/or oxidative damage.

Our previous work showed that exposure to 1 µg/l BNF + 100 µg/l ANF mimicked the AHR-regulated gene induction of higher, single doses of BNF (50 and 100 µg/l), suggesting alterations in metabolism may occur in this mixture toxicity (Timme-Laragy *et al.*, 2007). Increasing BNF exposure resulted in a dose-response induction of gclc, gstp, gpx1, and sod2 ($p \leq 0.024$), but not sod1 (Fig. 2). The highest dose of BNF (100 µg/l) yielded either similar (for gstp and gpx1) or lower levels than the combination dose.

Total GSH Quantification

Total GSH was measured at 72 hpf, a time period which followed pretreatment with NAC or BSO (24–48 hpf) and was 24 h postadministration of BNF + ANF at 48 hpf. Exposures to BNF, ANF, and BNF + ANF elicited no significant differences in GSH concentrations among groups (Fig. 3). However, all three of these exposures trended toward an increase in total
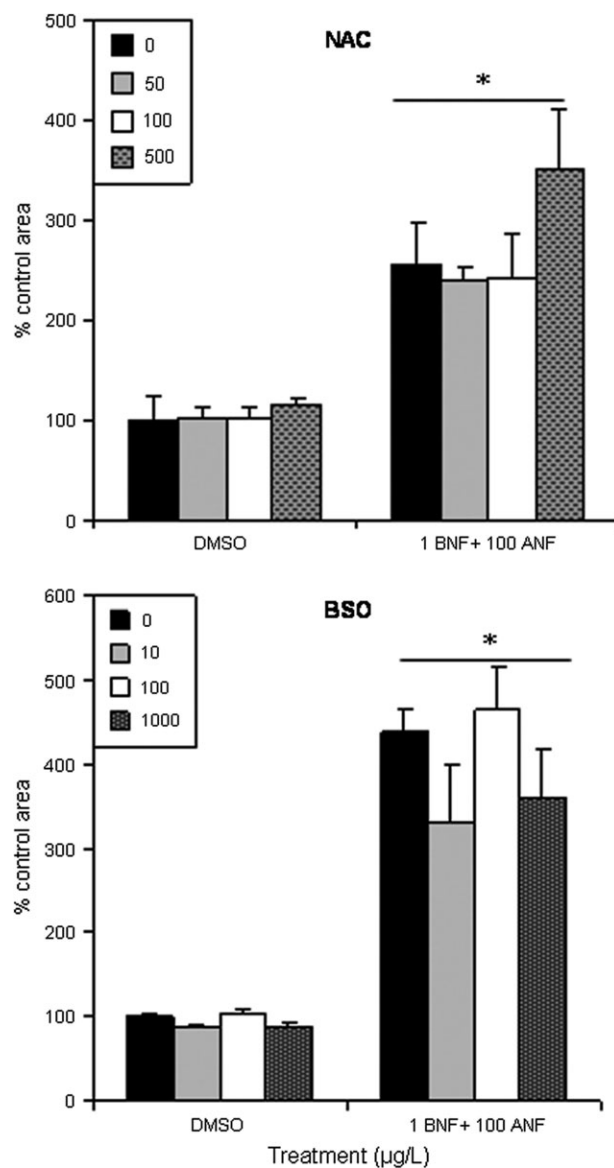

FIG. 4. Pretreatment with varying doses (µM) of NAC did not rescue pericardial effusion, and BSO did not worsen it. Pericardial effusion areas in BNF + ANF exposures are significantly higher than DMSO controls (*$p \leq$ 0.05). Single doses of 1 µg/l BNF and 100 µg/l ANF showed no significant differences from DMSO controls and were excluded from graphs for simplicity, but included in statistical analyses. Data are presented as mean + SEM; $n = 3$ pools of five embryos, and each data point is representative of at least 30 embryos.

GSH levels from approximately 30–40 µmol GSH/µg protein compared to DMSO controls (Fig. 3, $p = 0.075$–0.093). Pretreatment with 100µM NAC, an antioxidant that increases available pools of cysteine and can increase GSH levels, did increase total GSH concentrations in control embryos, but did not elevate levels above those observed by PAH treatment. As GSH is normally present in high concentrations, this may present an inherent difficulty in detecting a statistically significant increase in these samples. Also, these experiments
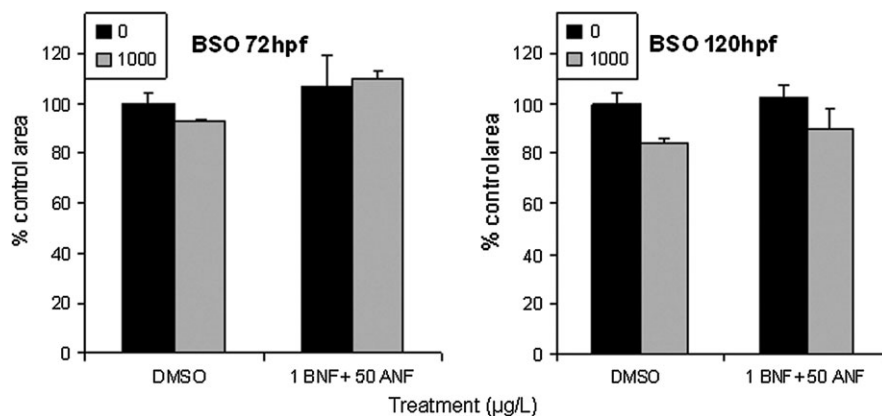
FIG. 5. Toxicity from a nontoxic combination of BNF + ANF is not exacerbated by pretreatment with 1000µM BSO at either 72 or 120 hpf. No statistically significant differences were observed in pericardial effusion between any of the treatment groups, including single exposure of 1 µg/l BNF and 50 µg/l ANF, which were excluded from the graph for simplicity. Data are presented as mean + SEM, $n = 3$ pools of five embryos.

measured total GSH in whole embryos, which could mask important tissue-specific changes.

BSO inhibits the rate-limiting enzyme in GSH synthesis, glutamate-cysteine ligase, thereby preventing replacement of GSH pools and hindering the GSH response to oxidative stress. BSO alone did not decrease GSH in the control treatments, but significant lower total GSH levels were observed with BSO and BNF and BNF + ANF groups compared with no pretreatment (Fig. 3, $p = 0.001$), indicating an accelerated depletion of GSH and increased utilization of this endogenous antioxidant peptide. This supports the hypothesis that oxidative events are occurring in this toxicity, although this may be a BNF effect that is not specific to the synergistic toxicity.

Redox Modulation and Deformities

In these experiments, embryos were pretreated with a redox-modulating chemical to determine whether a more reduced or oxidized redox level would alter deformity severity resulting from exposure to BNF + ANF. Pretreatment with NAC at nontoxic doses of 50 and 100µM did not prevent deformities, and 500µM worsened pericardial effusion in the BNF + ANF–treated embryos (Fig. 4). Controls treated with 500µM NAC began to show signs of toxicity as indicated by a slight increase in pericardial effusion and thinning of the lower jaw tissue. The highest dose used, 1000µM, was fatal (data not shown).

Pretreatment with BSO failed to worsen deformities at all nontoxic doses used (10, 100, and 1000µM) (Fig. 4). Furthermore, studies which used a nontoxic combination of BNF + ANF (1 µg/l BNF and 50 µg/l ANF) previously shown to exhibit toxicity upon injection of a CYP1A-morpholino (Billiard *et al.*, 2006), did not reveal deformity exacerbation by GSH depletion (Fig. 5).

NRF2 Knockdown and tBOOH Survival

Although the NRF2 morpholino sequence has been previously described and verified (Kobayashi *et al.*, 2002), those

experiments did not examine the effect of NRF2 knockdown on embryo survival to a prooxidant exposure. As did Kobayashi *et al.* (2002), we observed that injection of NRF2 morpholino at concentrations of 0.1 or 0.25mM had no effect on normal embryonic development (data not shown). In challenging NRF2- versus control morpholino–injected embryos with the model prooxidant tBOOH, we observed a statistically significant shift in the 48-h LC_{50} at 96 hpf from 891µM in control morpholino to 586µM in NRF2 morpholino–injected embryos (Table 3). This indicates that knockdown of NRF2 at this concentration (0.1mM) and injection volume (3–5 nl) is sufficient to render the fish more sensitive to the toxic effects of a prooxidant.

NRF2 Knockdown and Gene Expression

We also examined the effectiveness of the NRF2 morpholino to knock down several antioxidant genes following chemical exposure. Following one dose of 500µM tBOOH at 24 hpf and then again 3 h prior to sampling at 48 hpf (no death or deformities were observed at this time point), noninjected and control morpholino embryos exposed to tBOOH showed significant upregulation of gstp, gpx1, and gclc at levels indistinguishable from one another (Fig. 6). None of these genes

TABLE 3
Forty-Eight Hour LC_{50s} at 96 hpf and Confidence Intervals for Noninjected, Control Morpholino–Injected, and NRF2 Knockdown Embryos Exposed to the Prooxidant tBOOH at 48 and 72 hpf

	LC_{50} (mM tBOOH)	95% Confidence intervals
Noninjected	0.816	(0.675, 0.948)
Control morpholino	0.891	(0.735, 1.095)
NRF2 morpholino	0.586*	(0.459, 0.705)

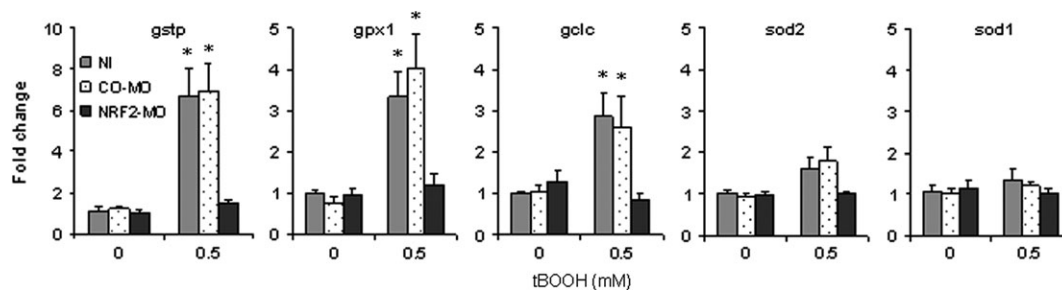Significant difference (*) from injected control.

FIG. 6. Induction of gene expression by tBOOH is inhibited by NRF2 knockdown in 48 hpf embryos exposed to tBOOH at 24 hpf and again 3 h prior to fixation. *$p \leq 0.05$.

were induced in tBOOH-exposed NRF2-MO embryos (Fig. 6). Following exposure of control and NRF2 morpholino–injected embryos to DMSO, 1 μg/l BNF, 100 μg/l ANF, or a BNF + ANF combination, there was a significant interaction between treatment and morpholino ($p \leq 0.003$) and significant induction of all five genes examined in the control morpholino groups exposed to BNF + ANF (sod1, sod2, gstp, gpx1, and gclc; Fig. 7, $p \leq 0.0035$). The induction of these genes was also significantly inhibited by NRF2 knockdown (Fig. 7, $p \leq 0.0008$).

NRF2 Knockdown and Deformities

Having confirmed the effectiveness of the morpholino in its ability to render the embryo more sensitive to oxidative stress and inhibit antioxidant gene induction, we then tested the hypothesis that NRF2 plays a protective role in the synergistic developmental toxicity of PAHs. We predicted that knockdown of NRF2 would exacerbate deformity severity caused by toxicity of BNF + ANF. Using a combination of BNF (1 μg/l) and ANF (50 μg/l) that does not cause pericardial effusion in noninjected embryos, we examined whether knockdown of NRF2 was able to exacerbate toxicity. NRF2 knockdown exacerbated pericardial effusion by 35% with the lower dose of ANF in combination with BNF ($p \leq 0.05$, Fig. 8), indicating that a fully functional antioxidant response utilizing NRF2 is protective against synergistic developmental toxicity of BNF + ANF.

DISCUSSION

We previously demonstrated that the AHR mediates the synergistic embryotoxicity of BNF + ANF and that CYP1A is protective (Billiard et al., 2006). To further understand the mechanism of this toxicity, we looked for genes downstream of the AHR besides cytochrome P450s that could play a role. NRF2 is one such candidate, with three AHR-binding xenobiotic response elements (XREs) in its promoter in the mouse, and it has been shown to be upregulated by TCDD (Miao et al., 2005). The current study demonstrates that antioxidant responses are acomponent of PAH synergistic developmental toxicity and that

NRF2 is protective against prooxidant and PAH exposures during development.

We have shown that antioxidant genes sod1, sod2, gstp, gpx1, and gclc are upregulated at 48 hpf after coexposure to 1 μg/l BNF + 100 μg/l ANF, but not affected by either of these doses alone. These data indicate that there are signaling pathways sensitive to redox perturbations that are responding to BNF + ANF prior to deformity onset at 48 hpf. This appears to be a transient response since at 96 hpf, following onset of pericardial effusion and craniofacial malformations in the BNF + ANF treatments, expression of these genes had returned to control levels. This is in contrast to AHR-regulated genes which remain elevated at this time point (Timme-Laragy et al., 2007).

The induction of antioxidant genes in this synergistic toxicity occurred without a significant change in total GSH, one of the most abundant cellular defenses against oxidative stress and an important cofactor in phase II metabolism. Other studies have observed increased GSH by PAH exposure in fish (Meyer et al., 2003; Wassenberg, 2004). We did significantly increase total GSH levels with pretreatment of NAC in controls, although NAC pretreatment did not exceed the levels of induction by the treatments with BNF and ANF. This could be due to the fact that GSH itself is a negative regulator of the modifier subunit of glutamate-cysteine ligase, the rate-limiting enzyme of GSH synthesis (Di Giulio and Meyer, 2008), thus maintaining a limit on maximum GSH content. Pericardial effusion could also not be reduced with NAC. Rather, at high concentrations, NAC appeared to actually exacerbate deformities.

GSH levels in another fish model, the channel catfish, have been shown to be difficult to modulate by BSO alone, requiring an additional stressor to deplete total GSH concentrations (Gallagher et al., 1992). Similarly, only in our BSO pre-treatment groups that were exposed to the additional stressor of BNF or BNF + ANF showed depletion of GSH, evidence that oxidative events are occurring. This may be a BNF effect that is not specific to the synergistic toxicity, further suggested by the lack of GSH depletion on deformity severity.

Some prooxidants have been shown to cause similar defor-mities as those produced by BNF + ANF in early life stages of
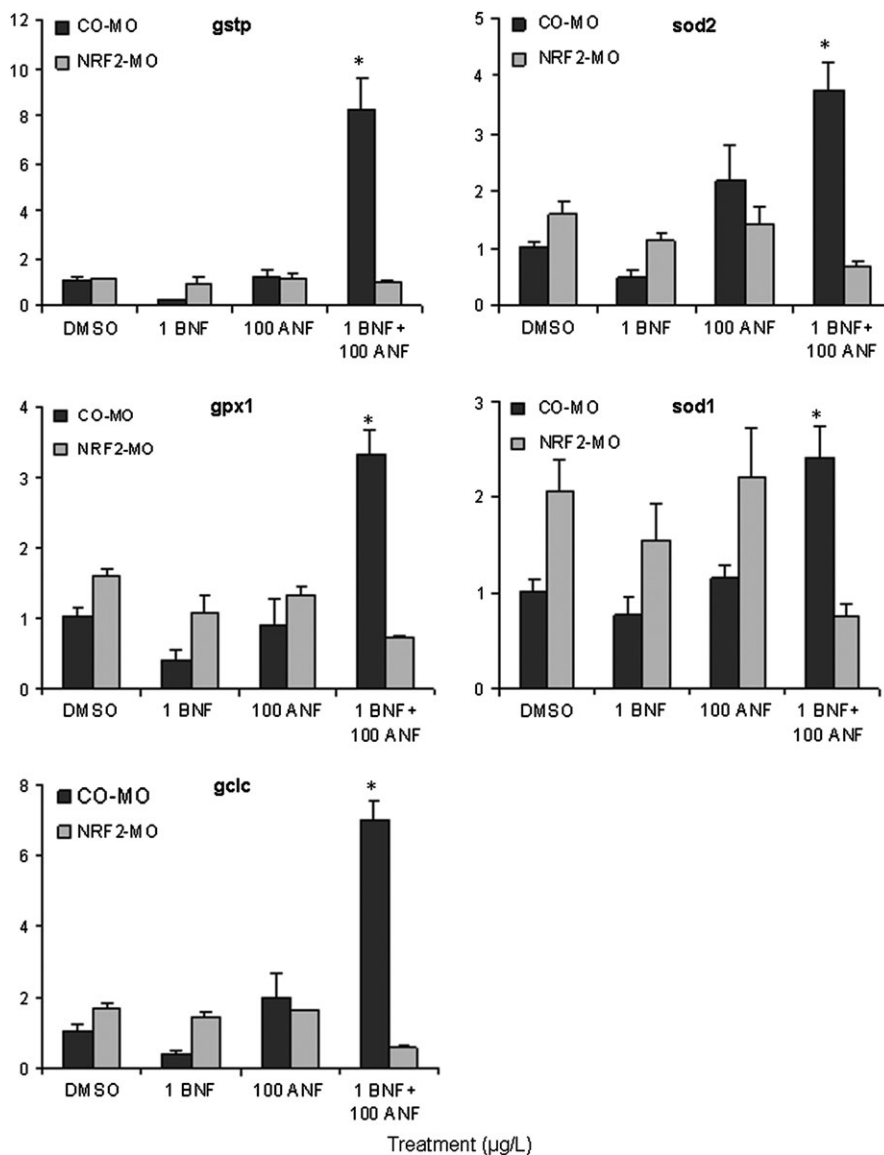
FIG. 7. Inhibited upregulation of antioxidant genes at 48 hpf in NRF2 knockdown (NRF2-MO) compared to control morpholino (CO-MO)–injected embryos after a 24-h exposure to DMSO, 1 μg/l BNF, 100 μg/l ANF, or a combination of BNF + ANF. There was a significant interaction between injection and treatment ($p \leq 0.003$) in two-way ANOVAs. (*$p \leq 0.004$; mean + SEM, $n = 3$).

fish, such as cumene hydroperoxide in killifish (Wassenberg, 2004) and ethanol in zebrafish (Reimers *et al*., 2004). Yet the absence of deformities in our nontoxic NAC and BSO pretreatment experiments does not mean that oxidative stress is not an important factor in BNF + ANF toxicity. Oxidative stress does not always result in gross deformities. For example, the prooxidant paraquat failed to produce deformities in rainbow trout larvae (Bauder *et al*., 2005), and we also did not observe any deformities with the prooxidant tBOOH.

Perturbed redox signaling could contribute to deformities in ways that are independent of thiol status (Jones, 2006b). Beyond GSH status, specifically that of GSH:GSSG which was not a measurement parameter in our experiments, other important thiol/disulfide antioxidant compartments exist: cysteine/cystine in the extracellular space, and thioredoxin-1 (-SH$_2$/-SS-). All three of these are independently controlled and do not necessarily equilibrate amongst each other (Hansen *et al*., 2006; Jones, 2006a,b; Jones *et al*., 2004). Methods by which to evaluate these redox ratios in zebrafish embryos would be very useful, but to our knowledge, are not yet available. We would predict that as we were able to detect changes in the coarse measurement of total GSH with BSO pretreatment and BNF exposure, if we were able to examine these three redox compartment ratios we may yet see more significant changes indicative of oxidative
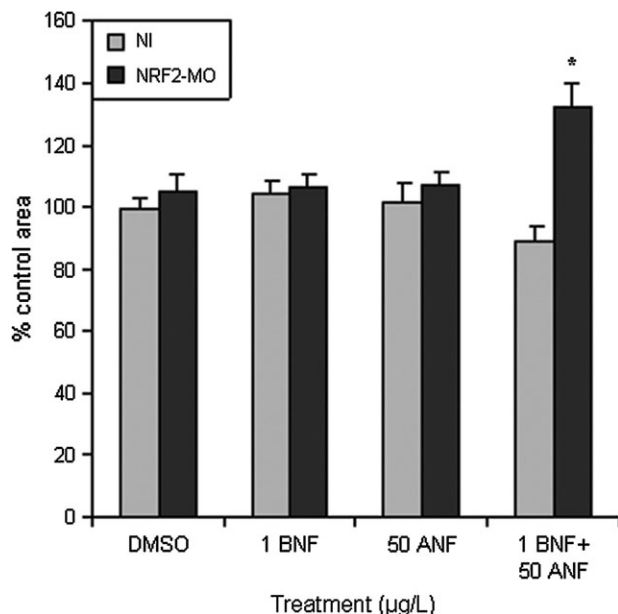
FIG. 8. Quantified pericardial effusion at 96 hpf. This dose does not cause pericardial effusion in noninjected (NI) fish, but in NRF2 morpholino (NRF2-MO) embryos, toxicity is exacerbated and results in moderate pericardial effusion. Significant differences noted between both DMSO controls and NI treatment–matched group (*$p \leq 0.003$). Data are presented as mean + SEM, $n = 20$–30 fish.

stress. However, since ARE signaling can occur independent of GSH:GSSG status and of Trx-1 (Go *et al.*, 2004), changes in antioxidant gene expression are a sufficient method by which to capture significant perturbations in redox status.

To understand how antioxidant responses factor into deformities of this toxicity, we cast a wider net over the redox-responding capability of the embryo by knocking down the ARE transcription factor NRF2. In doing so, we prevented the upregulation of all antioxidant genes examined and also exacerbated pericardial effusion. However, this deformity was only mildly enhanced, suggesting that while oxidative stress is a component of this synergistic toxicity, its precise role in contributing to deformities remains unclear.

AREs are present in many phase II genes; XREs are found in both phase I and phase II genes (Rushmore and Kong, 2002). These two mediators of the xenobiotic detoxification system were previously thought to function independently, but recent identification of three functional XREs in the promoter region of NRF2 (Miao *et al.*, 2005) has caused this assumption to be reevaluated. Knockdown of AHR in mouse cell lines abolished NRF2 induction by TCDD. Site-directed mutagenesis showed that both the ARE and XRE in the NRF2 promoter region can result in mRNA induction of NRF2 by TCDD, and the AHR was shown to directly bind to the NRF2 promoter (Miao *et al.*, 2005). Mouse, rat, and human NRF2 promoters all have multiple copies of XREs (Miao *et al.*, 2005). Little is known about this cross-talk in fish models.

There are several genes with both XRE and AREs including sod1, nqo1, ugt1a, and gstα (Nguyen *et al.*, 2003b). In addition, some chemicals, including the PAHs BNF and benzo(a)pyrene, have been designated as bifunctional inducers, by which metabolism via AHR-XRE–regulated induction of CYP1A can form reactive metabolites that lead to induction of NRF2-ARE–regulated genes (Nguyen *et al.*, 2003b). Our gene expression data showed that the antioxidant genes examined were not induced in NRF2 knockdown embryos; similar results have also been shown in the mammalian and piscine literature (Kobayashi and Yamamoto, 2005; Kobayashi *et al.*, 2004; Rushmore and Kong, 2002; Zhu *et al.*, 2005). The fact that NRF2 knockdown prevented upregulation of these genes with exposure to both PAHs and a prooxidant suggests that the ARE is the more important response element in these promoters.

Knocking down NRF2 increased sensitivity to the tBOOH (decreased LC_{50}) as well as to synergistic PAH toxicity (enhanced pericardial effusion). Other studies also support a protective role for NRF2 in ROS and PAH toxicity. In NRF2−/− mice, cardiac fibroblasts from newly born pups have been shown to have lower expression/antioxidant activity of catalase, glutathione-*s*-transferase, NAD(P)H quinine oxidoreductase 1, glutathione reductase, and lower GSH levels (Zhu *et al.*, 2005). These cells were also more sensitive to cytotoxic effects of ROS and reactive nitrogen species. Further evidence is seen in adult NRF2−/− mice, which are more susceptible to oxidative damage from PAHs and diesel exhaust. These mice have been shown to have increased rates of stomach neoplasia following exposure to benzo(a)pyrene (Ramos-Gomez *et al.*, 2001) and accelerated 8-oxo-DG DNA adduct formation in lungs of mice exposed to diesel exhaust (Aoki *et al.*, 2001).

In conclusion, we show antioxidant responses, which can be taken as an indication of oxidative stress, are a component of the synergistic developmental toxicity of BNF + ANF and also specify a protective role for NRF2 in prooxidant and PAH exposures. However, the contribution of oxidative stress to the development of related deformities remains unclear. Additional studies are necessary to further an understanding of the role of oxidative stress in the developmental toxicity of PAHs in early life stages of fishes and possibly other vertebrates.

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