TOXICOLOGICAL SCIENCES **115(2)**, 391–400 (2010)
doi:10.1093/toxsci/kfq066
Advance Access publication March 3, 2010

The Role of Nrf2 and MAPK Pathways in PFOS-Induced Oxidative Stress in Zebrafish Embryos

Xiongjie Shi and Bingsheng Zhou[1]

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

[1] To whom correspondence should be addressed. Fax: +86-27-68780123. E-mail: bszhou@ihb.ac.cn.

Received November 4, 2009; accepted February 25, 2010

Perfluorooctane sulfonate (PFOS) is a persistent organic pollutant and causes oxidative stress, apoptosis, and developmental toxicity in zebrafish embryos. In the present study, we examined nuclear factor erythroid 2–related factor 2 (Nrf2)– and mitogen-activated protein kinases (MAPKs)–mediated oxidative stress pathways in zebrafish embryos upon exposure to PFOS. Four-hour postfertilization (hpf) zebrafish embryos were exposed to 0.2, 0.4, and 1.0 mg/l PFOS until 96 hpf. PFOS enhanced production of reactive oxygen species (ROS) in a concentration-dependent manner. Activity of antioxidative enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, was significantly induced in zebrafish larvae in all PFOS-treated groups relative to the control. Exposure to 1.0 mg/l PFOS significantly increased malondialdehyde production in zebrafish larvae. The Nrf2 and heme oxygenase-1 (HO-1) gene expressions were both significantly upregulated compared with the control group. For MAPKs, we investigated gene expression profiles of extracellular signal–regulated protein kinase (ERK), c-Jun NH (2)-terminal kinase (JNK), and p38. The ERK gene expression levels were unchanged, whereas JNK and p38 gene expressions were significantly upregulated, which could be linked to PFOS-induced cell apoptosis in zebrafish larvae. In addition, we found that coexposure with sulforaphane, an Nrf2 activator, could significantly protect against PFOS-induced ROS generation, whereas inhibition of MAPKs did not exhibit significant effects on PFOS-induced HO-1 gene expression and ROS production. Furthermore, we showed that morpholino-mediated knockdown of Nrf2 reduced PFOS-induced HO-1 gene expression. These findings demonstrate that Nrf2 is protective against PFOS-induced oxidative stress in zebrafish larvae.

Key Words: **PFOS; ROS; oxidative stress; Nrf2; MAPKs; zebrafish.**

Many environmental pollutants can generate reactive oxygen species (ROS) and oxidative stress and have a profound impact on the survival of organisms (Livingstone, 2001). However, most cells have evolved intricate mechanisms to prevent generation of ROS or detoxify ROS via activation of a family of antioxidant/detoxification enzymes that enhance cellular ROS scavenging capacity in maintaining cellular redox homeostasis and reducing oxidative damage (Copple *et al.*, 2008; Kobayashi and Yamamoto, 2006). There have been increasing lines of evidence that show that oxidative stress activates transcription of a variety of antioxidant genes governed by key signaling pathways involved in cellular oxidative responses (Sen and Packer, 1996). Among these, nuclear factor erythroid 2–related factor 2 (Nrf2) has been demonstrated to be a critical transcription factor that binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for phase I and phase II antioxidative enzymes and cytoprotective proteins (Nguyen *et al.*, 2009). Hence, Nrf2 is presumably one of the most important pathways for cells to deal with oxidative stress generated from exposure to exogenous and endogenous chemicals (reviewed by Kensler *et al.*, 2007; Niture *et al.*, 2009; Pi *et al.*, 2008).

Among the various cytoprotective enzymes, e.g., heme oxygenase-1 (HO-1) has been shown to play a key role in maintaining antioxidant homeostasis during cellular stress (Alam and Cook, 2003; Hong *et al.*, 2009; Hsieh *et al.*, 2008). In addition, the induction of HO-1 gene expression is primarily regulated at the transcriptional level, and its inducibility by various inducers is linked to the transcription factor Nrf2 (Alam and Cook, 2003), whereas Nrf2 nuclear translocation requires the activation of several signal transduction pathways, including mitogen-activated protein kinases (MAPKs) (Shen *et al.*, 2004). MAPKs are downstream effectors in antioxidant responses, and their activities are manifested in activation of many transcription factors, including Nrf2 (Limón-Pacheco *et al.*, 2007). Hence, Nrf2 is an important transcriptional regulator of numerous detoxifying and antioxidant genes (Niture *et al.*, 2009; Pi *et al.*, 2008), and MAPK plays a key role in transducing various extracellular signals to the nucleus (Johnson and Lapadat, 2002). Three subfamilies of MAPKs have been described, including extracellular signal–regulated protein kinase (ERK), c-Jun NH(2)-terminal kinase (JNK), and p38 MAPK (Johnson and Lapadat, 2002). ERK2 and p38 MAPK also positively regulate

Nrf2 activity to initiate the transcription of antioxidant genes (Zipper and Mulcahy, 2000). *In vitro* models have shown that extracellular regulated ERK2 and p38 MAPK modulate the expression of antioxidant enzymes by activation of Nrf2.

Perfluorooctane sulfonate (PFOS) is a major constituent of commercial perfluorinated compounds (PFCs) and widely used as surfactants in industrial and consumer applications (Giesy and Kannan, 2001). In addition, PFOS appears to be the ultimate degradation product of a number of commercially produced PFCs (Lau *et al*., 2007). Monitoring studies have found that PFOS is the predominant compound in the environment and in various organisms, including fish worldwide (Giesy and Kannan, 2001; reviewed by Lau *et al*., 2007). PFOS has been known to biomagnify through food chains and oviparous transfer in fish (Ankley *et al*., 2005; Kannan *et al*., 2005). The properties of its highly persistent biomagnification have generated a notable interest in its potential toxicity. In May 2009, PFOS was listed under the Stockholm Convention among the persistent organic pollutants in Geneva.

Exposure to PFOS has been shown to induce various toxic effects in fish, such as hepatotoxicity (Hagenaars *et al*., 2008) and developmental and reproductive toxicity (Ankley *et al*., 2005; Oakes *et al*., 2005; Shi *et al*., 2008). A recent study reported that exposure of PFOS to carp altered expression of genes mainly involved in energy metabolism, reproduction, and stress response in the liver (Hagenaars *et al*., 2008). In zebrafish embryos/larvae, PFOS exposure caused cellular apoptosis and alteration of certain gene expressions related to cellular apoptosis (e.g., p53 and Bax) (Shi *et al*., 2008) and significantly induced the protein expression of peroxiredoxin 2, which is thought to be involved in cellular defenses against oxidative stress (Shi *et al*., 2009). These observations suggested that oxidative damage may play an important role in developmental toxicity by PFOS.

In fish, ROS-induced oxidative stress is thought to contribute to abnormal development during embryogenesis (Yamashita, 2003). Fish embryonic development may be especially sensitive to toxicant-induced oxidative stress since even a 15–20% increase in ROS can tip progenitor cells into premature cell cycle arrest or differentiation (Li *et al*., 2007). Hence, toxicant exposures that cause oxidative stress during embryonic development are a significant health risk in adults (Timme-Laragy *et al*., 2009). Although it is well established that PFOS induces oxidative damage in cultured cells and in fish (Huang *et al*., 2009; Krøvel *et al*., 2008; Liu *et al*., 2007a; Shi *et al*., 2008; Wei *et al*., 2009), there has been no assessment as to whether Nrf2 and MAPKs are involved in the oxidative stress response induced by PFOS. In the present study, by using zebrafish embryos, we investigated the effects of PFOS on the Nrf2- and MAPK-mediated antioxidant responses. The formation of ROS and changes in the activities of antioxidative enzymes and lipid peroxidative product were measured. To evaluate whether the oxidative stress would induce particular cell signaling pathways at early developmental stages, gene expressions of the Nrf2 and MAPKs pathways (i.e., ERK, JNK, and p38) were examined. We also used sulforaphane (SFN) to activate Nrf2 gene expression and chemically inhibited MAPKs to assess the effects on HO-1 gene expression and ROS production with or without SFN treatment. Gene knockdown of Nrf2 was further used to identify a protective role for Nrf2 in embryonic oxidative stress.

MATERIALS AND METHODS

Chemicals. Heptadecafluorooctanesulfonic acid potassium salt (PFOS, > 99%) was obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). The stock solution (50,000 mg/l) was prepared by dissolving the crystals in high-performance liquid chromatography–grade dimethyl sulfoxide (DMSO) and storing it at 4°C. SFN was purchased from Sigma Chemical Corporation (St Louis, MO). PD98059, SP600125, and SB203580 were purchased from Beyotime Institute of Biotechnology (Shanghai, China). All the other chemicals and reagents were of analytical grade.

Zebrafish maintenance and embryo exposure. The wild-type (AB strain) zebrafish were maintained at 28 ± 0.5°C in a 14-h light/10-h dark cycle in a continuous flow-through system in charcoal-filtered tap water. The fish were fed with live *Artemia nauplii* twice daily. Fertilized eggs were obtained from natural mating of adult zebrafish in our laboratory as previously described (Shi *et al*., 2008). Zebrafish eggs were collected within 4 h of spawning from several breeding tanks, pooled, washed, and then randomly transferred into a glass beaker containing 500 ml of exposure solution (0, 0.2, 0.4, and 1.0 mg/l) of PFOS (~300 eggs per beaker). In coexposure treatments, each PFOS exposure group and control group were also cotreated with 40μM SFN, an activator of Nrf2 (Kobayashi *et al*., 2009). To inhibit the MAPKs pathway, zebrafish embryos were exposed to 1μM of ERK-MAPK–specific inhibitor (PD98059), JNK-MAPK–specific inhibitor (SP600125), or p38-MAPK–specific inhibitor (SB203580) (Liu *et al*., 2007b) with or without SFN. Both the control and the treated embryos received 0.03% (vol/vol) DMSO. Each treatment was performed in four replicates. The beakers were kept in a humidified incubator at 28.5 ± 0.5°C under controlled lighting conditions.

Detection of ROS and antioxidative enzyme assay. At 96-h postfertilization (hpf), ~200 zebrafish larvae were homogenized in 800 μl CytoBuster protein extraction buffer (Novagen, San Diego, CA) using an Ultra-Turrax T8 basic homogenizer (IKA, Staufen, Germany). The homogenates were centrifuged at 12,000 × g for 10 min in 4°C, and the supernatants were collected for various assays. ROS concentrations were assessed using the oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich, St Louis, MO) as previously described (Deng *et al*., 2009). The fluorescence intensity was measured using a microplate reader (Molecular Device, M2, Union City, CA) with excitation and emission at 485 and 530 nm, respectively. The ROS concentration was expressed in arbitrary units (DCF/mg protein).

Glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) activities were measured according to the protocols of commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). GPx activity was measured by the dithio nitrobenzene method and was measured at 412 nm (Griffith, 1980). The level of GPx is expressed as units per milligrams of protein. CAT activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption as previously described (Luck, 1963). The level of CAT is expressed in terms of micromoles H_2O_2 consumed per minute per milligram of protein. SOD activity was evaluated by the inhibition of the rate of the superoxide radicals–dependent cytochrome C reduction, and one unit of activity is defined as the amount of protein necessary to decrease the reference rate to 50% of maximum inhibition (Flohé

and Otting, 1984). The results of this enzymatic assay are given in units of SOD activity per milligram of protein (U/mg), where 1 U of SOD is defined as the amount of sample causing 50% inhibition of cytochrome C reduction.

Lipid peroxidation and protein assay. Lipid peroxidation was detected as malondialdehyde (MDA) reacting with thiobarbituric acid to form a colored complex by spectrofluorometric analysis according to the method described by Ohkawa *et al.* (1979). The level of MDA is expressed as nanomoles per milligram protein. Protein concentrations were measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ).

RNA extraction. At 96 hpf, 30 zebrafish larvae were selected randomly from each beaker, placed in RNase-free Eppendorf tubes and then homogenized in 1 ml ice-cold Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted following the manufacturer's instructions, followed by treatment with RNase-free DNase I (Promega, Madison, WI) to remove any remaining genomic DNA. The concentration of total RNA was measured based on the A260 value. The purity of the RNA in each sample was verified by determining the A260/A280 ratio and then visually confirming 1.0 μg RNA on 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining.

Quantitative real-time PCR assay. Synthesis of the complementary DNA was performed using 2 μg total RNA from each sample mixed with 500 ng of random primers (Takara, Kyoto, Japan) using M-MLV Reverse Transcriptase (Promega), as described by the manufacturer. Quantitative real-time PCR was carried out using a SYBR Green PCR Kit (Bioteke, Peking, China), and PCR amplification was conducted on an ABI PRISM 7300 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA). The gene names, GenBank accession numbers, forward and reverse primer sequences, and amplicon sizes are listed in Table 1. Each reaction was performed in three replicate samples. The expression level of each target gene was normalized to its 18S messenger RNA (mRNA) content. After verifying that the amplification efficiencies of the primers for the selected genes and 18S mRNA are approximately equal, the fold differences in the expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Embryo morpholino injection. Nrf2 gene was knocked down using the MO technique as described by Timme-Laragy *et al.* (2009). Zebrafish Nrf2 gene translation–blocking morpholino (Nrf2-MO) 5′-CATTTCAATCTCCATCATGTCTCAG-3′ and a standard control morpholino (MO-control) 5′-CCTCTTACCTCAGTTACAATTTATA-3′ were obtained from Gene Tools, LLC (Philomath, OR). All MOs were dissolved in distilled water at a concentration of 3mM and diluted at a concentration of 0.1mM in 1× Danieau Media (58mM NaCl, 0.7mM KCl, 0.4mM MgSO4, 0.6mM $Ca(NO3)_2$, and 5.0mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6). Prior to microinjection, the solutions were heated for 5 min to 65°C and then kept at 37°C prior to use. The morpholinos were injected into embryos at the one- to four-cell stage with ~3 nl of morpholino using a Harvard Apparatus PLI-100 (Cambridge, MA). Noninjected, MO-control, and Nrf2-MO embryos were exposed to concentrations of 0, 0.2, 0.4, and 1.0 mg/l PFOS with or without SFN cotreatment.

TABLE 1
Primer Sequence for the Quantitative Reverse Transcription PCR used in this Study

Name	Gene bank accession No.	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	Product length (bp)
18S RNA	NM_200713	ttgttggtgttgttgctggt	ggatgctcaacagggttcat	136
ERK2	AY922320	gttgaagacgcagcacttga	acaggtttgatggcttcagg	122
ERK3	DQ360074	cagggctttgacttcgactc	tgtgaaacggaggtgttca	107
JNK1	AB030900	acaggaataagcgcgagaaa	tggtcatacgctgagcagac	133
p38a	AB030897	actcgcattccaagcagact	gttgtcttgcaaacgctcaa	133
p38b	AB030898	cggagcggtaccagaattta	cgggagagtttcttcactgc	109
Nrf2	AB081314	gacaaaatcggcgacaaaat	ttaggccatgtccacacgta	165
HO-1	NM_199678	ggaagagctggacagaaacg	cgaagaagtgctccaagtcc	107

Statistical analysis. The homogeneity of variances was checked with Levene's test, and the differences were evaluated by one-way ANOVA followed by Tukey's test using SPSS 13.0 (SPSS, Chicago, IL). All the data are expressed as the mean ± SEM. A p-value of < 0.05 is considered statistically significant.

RESULTS

ROS Measurement

In PFOS-treated zebrafish embryos, ROS concentrations were significantly increased by 24.2 and 29.5% in the 0.4 and 1.0 mg/l treated groups, respectively, compared with the control (Fig. 1A). On the other hand, coexposure with SFN did not exhibit significant hypergeneration of ROS in zebrafish larvae in all chemically exposed groups (Fig. 1A). Induction of ROS with PFOS exposure was not observed in the SFN-treated groups.

Activities of Antioxidative Enzyme

In PFOS-treated zebrafish embryos, SOD activity was significantly increased in a concentration-dependent manner by 29.3, 40.6, and 42.6% in the 0.2, 0.4, and 1.0 mg/l exposure groups, respectively, relative to the control group (Fig. 1B). A significant increase in GPx activity was observed in all the exposure groups compared with the control (81.6, 135.8, and 96.8%) (Fig. 1B). CAT activity was increased by 45.2, 113.5, and 169.7% in the 0.2, 0.4, and 1.0 mg/l PFOS exposure groups, respectively, compared with the control groups (Fig. 1B).

Lipid Peroxidation

MDA was assessed as an index of lipid peroxidation. The levels of MDA were slightly increased but not significantly changed in the 0.2 and 0.4 mg/l PFOS exposure groups, whereas exposure to 1.0 mg/l PFOS caused a significant increase in MDA production in zebrafish larvae (Fig. 1C).

Gene Expression Profile

Exposure of the Nrf2 activator (SFN) alone significantly upregulated Nrf2 gene expression compared with the DMSO control group (Fig. 2A). The Nrf2 gene expression was significantly upregulated upon exposure to 0.4 and 1.0 mg/l PFOS by 2.5- and 3.0-fold, respectively, compared with the control group (Fig. 2A). Coexposure with SFN strongly induced Nrf2 gene expression by 3.6-, 4.0-, and 6.2-fold in the 0.2, 0.4, and 1.0 mg/l PFOS treatment groups, respectively (Fig. 2A). Similarly, SFN treatment alone also significantly upregulated HO-1 gene expression compared with the DMSO control group (Fig. 2B). HO-1 gene expression was

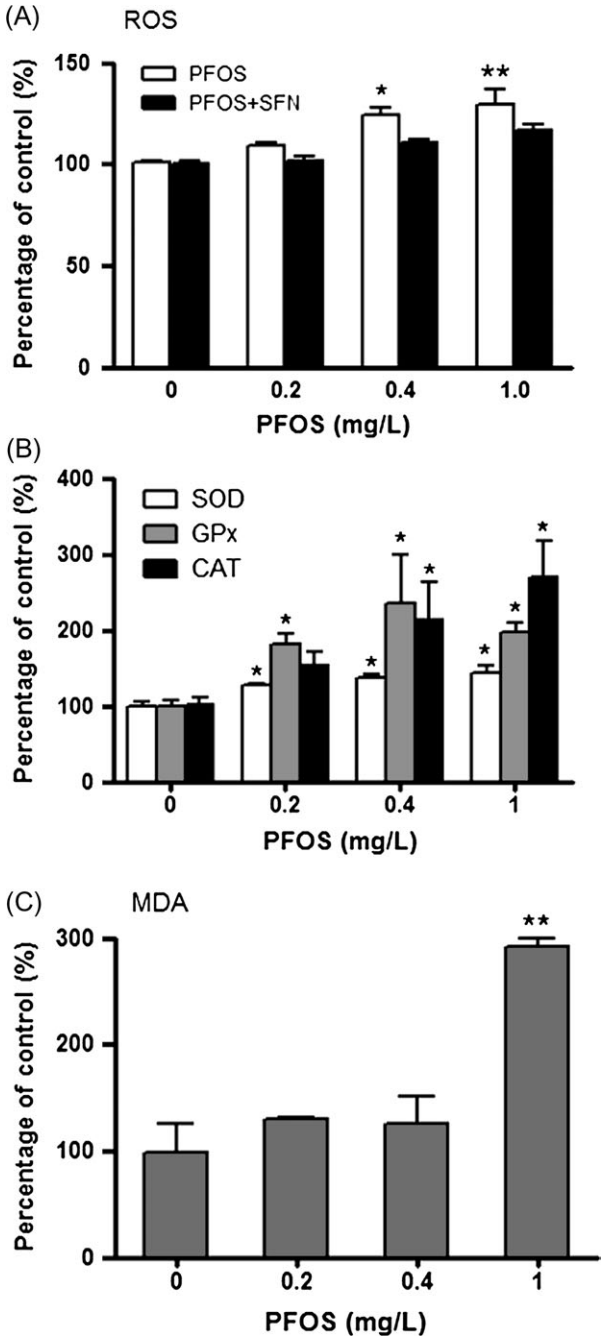

FIG. 1. Changes in ROS formation (A); changes in activities of SOD, PGx, and CAT (B); and changes in MDA concentrations (C) in the zebrafish larvae upon exposure to 0, 0.2, 0.4, and 1.0 mg/l PFOS or coexposure to 40μM SFN (A) until 96 hpf. Values that are significantly different from the vehicle control are indicated by asterisks (one-way ANOVA, $*p < 0.05$; $**p < 0.01$). Values are the means of four replicate exposures and are presented as mean ± SEM.

significantly upregulated in the 0.4 and 1.0 mg/l PFOS exposure groups with 2.8- and 3.0-fold changes, respectively (Fig. 2B). Coexposure with SFN strongly induced HO-1 gene expression by 3.4-, 5.5-, and 5.5-fold in the 0.2, 0.4, and 1.0 mg/l PFOS treatment groups, respectively (Fig. 2B).

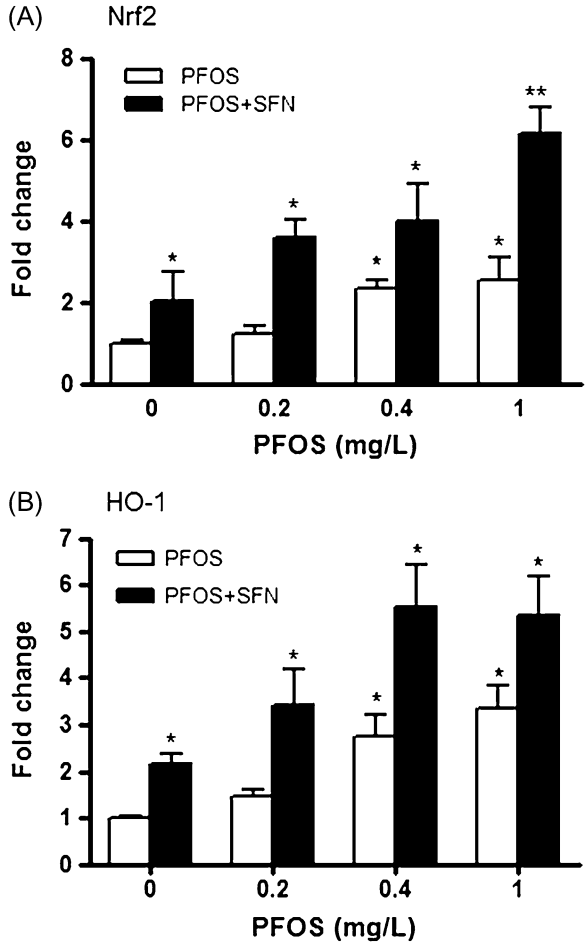

FIG. 2. Real-time quantitative PCR analyses of gene expression levels of Nrf2 (A) and HO-1 (B) in the zebrafish larvae upon exposure to 0, 0.2, 0.4, and 1.0 mg/l PFOS cotreatment with or without 40μM SFN until 96 hpf. Values that are significantly different from the vehicle control are indicated by asterisks (one-way ANOVA, $*p < 0.05$; $**p < 0.01$). Values are the means of three determinations on each of the three replicate exposures and are presented as mean ± SEM.

In zebrafish embryos, constitutive expressions of Erk2 and Erk3 could be detected throughout embryogenesis (Krens *et al.*, 2006). Therefore, in the MAPK pathway, we measured gene expressions of Erk2 and Erk3 at 96 hpf. The results showed that there were no significant differences in Erk2 and Erk3 gene expressions in any PFOS-treated groups compared with the controls (Fig. 3A and 3B). JNK1 gene transcription was significantly upregulated by 1.8-, 2.2-, and 2.9-fold in the 0.2, 0.4, and 1.0 mg/l PFOS exposure groups, respectively (Fig. 3C). In zebrafish, two isoforms of p38 (p38a and p38b) have been cloned (Fujii *et al.*, 2000). The p38a gene expression was significantly upregulated in a concentration-dependent manner at 1.6-, 2.1-, and 2.2-fold (Fig. 3D), whereas p38b gene expression was also significantly upregulated at 1.9-, 2.1-, and 3.7-fold changes in the 0.2, 0.4, and 1.0 mg/l PFOS exposure groups, respectively (Fig. 3E).

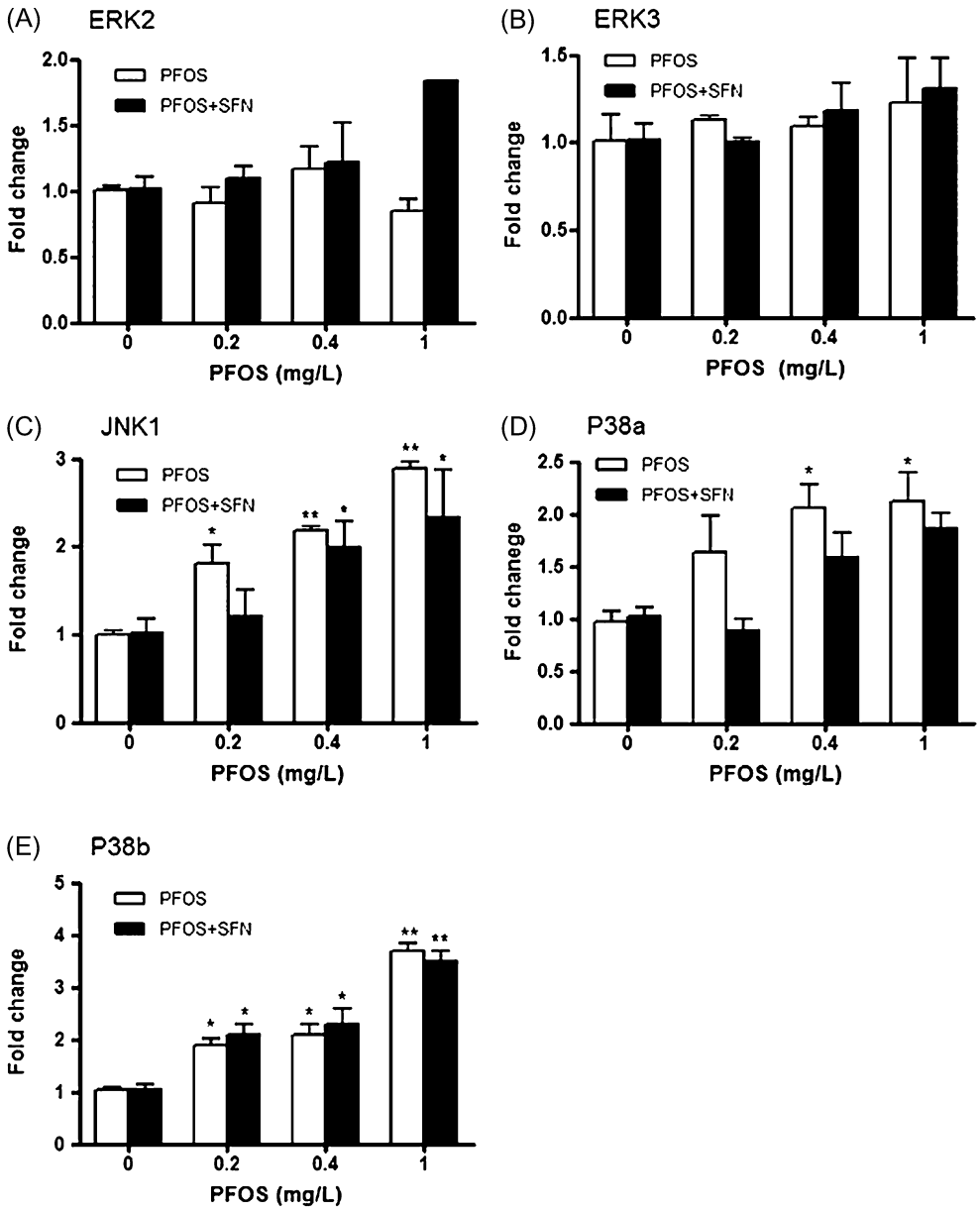

FIG. 3. Real-time quantitative PCR analyses of ERK2 gene expression levels (A), ERK3 (B), JNK1 (C), p38a (D), and p38b (E) in the zebrafish larvae upon exposure to 0, 0.2, 0.4, and 1.0 mg/l PFOS cotreatment with or without 40μM SFN until 96 hpf. Values that are significantly different from the vehicle control are indicated by asterisks (one-way ANOVA, $*p < 0.05$; $**p < 0.01$). Values are the means of three determinations on each of the three replicate exposures and are presented as mean ± SEM.

To evaluate the influence of SFN on the MAPK pathway, we also examined the effects of SFN treatment on MAPK gene expression. The results showed that with coexposure of SFN with DMSO, the ERK2, ERK3, JNK1, p38a, and p38b gene expression profile was not changed compared with the vehicle controls (Fig. 3A–E). However, cotreatment of zebrafish embryos with SFN and PFOS resulted in significant upregulation of JNK1 and p38b gene expressions as observed in the 0.4 and 1.0 mg/l PFOS exposure groups (Fig. 3C and 3E).

We further analyzed the effects of inhibitors of MAPKs on HO-1 induction and ROS production with or without SFN treatment. The results showed that with treatment of MAPK inhibitors, HO-1 gene expression was not affected relative to DMSO controls (Fig. 4A), whereas it was significantly upregulated in the 0.4 and 1.0 mg/l PFOS exposure groups (Fig. 4A). Inhibition of MAPKs did not affect PFOS-induced HO-1 gene expression relative to the groups without inhibitors (Fig. 4A). On the other hand, with cotreatment of MAPK inhibitors and SFN, the HO-1 gene expression was further

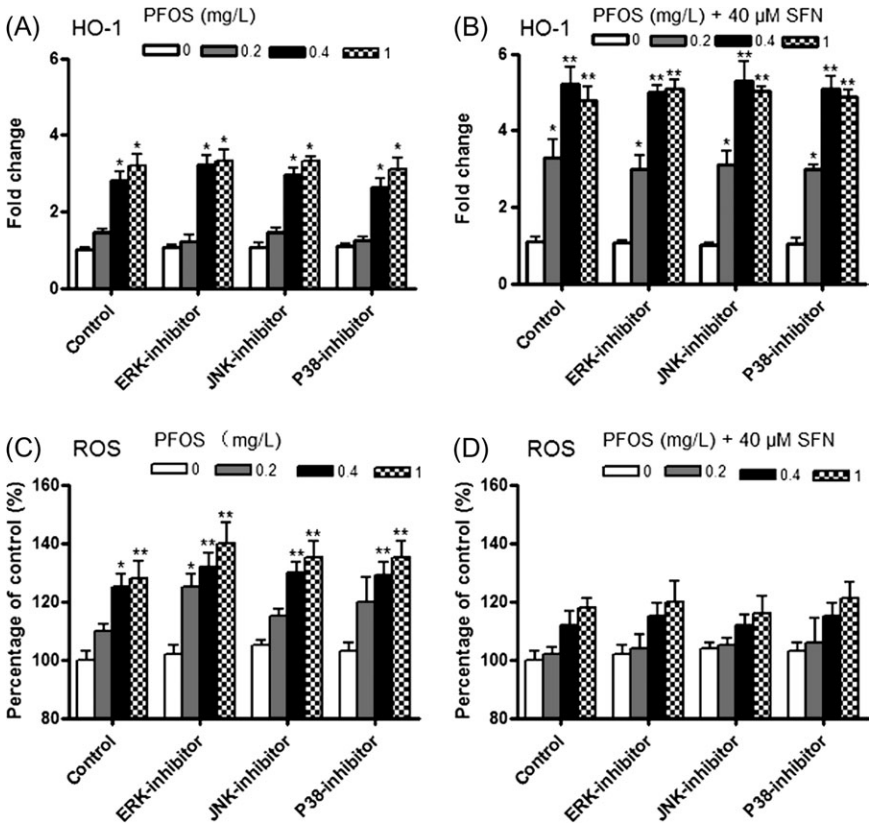

FIG. 4. Effects of inhibitors of MAPKs on HO-1 induction and ROS production in zebrafish larvae upon exposure to 0, 0.2, 0.4, and 1.0 mg/l PFOS cotreatment with or without 40μM SFN until 96 hpf: (A) real-time quantitative PCR analyses of gene expression levels of HO-1 in zebrafish exposure to PFOS without SFN; (B) real-time quantitative PCR analyses of gene expression levels of HO-1 in zebrafish coexposed to PFOS and SFN; (C) changes in ROS formation in zebrafish exposed to PFOS without SFN; and (D) changes in ROS formation in zebrafish coexposed to PFOS and SFN. Values that are significantly different from the control are indicated by asterisks (one-way ANOVA, $*p < 0.05$; $**p < 0.01$). Values are presented as mean ± SEM.

increased in the 0.2, 0.4, and 1.0 mg/l PFOS exposure groups (Fig. 4B). We also observed that treatment with MAPK inhibitors alone significantly increased ROS concentrations in PFOS exposure groups relative to respective control (Fig. 4C), but they did not affect PFOS-induced ROS production compared with those in the groups without inhibitors (Fig. 4C). However, upon cotreatment of SFN with MAPK inhibitors, there were no significant differences in ROS production among all the PFOS exposure groups (Fig. 4D).

Nrf2 Gene Knockdown

We examined the effectiveness of the Nrf2 morpholino knockdown on HO-1 gene expression and ROS generation with or without SFN treatment. Noninjected and control morpholino embryos exposed to 0.4 and 1.0 mg/l PFOS showed significant upregulation of HO-1 gene expression (Fig. 5A). The induction of HO-1 gene expression by PFOS exposure was significantly inhibited by Nrf2 knockdown (Fig. 5A). However, SFN treatment of embryos in which Nrf2 was knocked down by morpholino resulted in significantly upregulated HO-1 gene expression in the zebrafish larvae exposed to 1.0 mg/l PFOS (Fig. 5A). In the ROS assay, PFOS exposure (0.4 and 1.0 mg/l) induced a significant increase of ROS production in noninjected and morpholino control–treated larvae compared with DMSO control (Fig. 5B), whereas further hypergeneration of ROS in zebrafish larvae was observed in the Nrf2-morpholino knockdown groups by exposure to 0.2, 0.4, and 1.0 mg/l PFOS compared with all other groups (Fig. 5B). However, with coexposure to SFN, ROS production did not show any significant changes in the PFOS exposure groups (Fig. 5B).

DISCUSSION

In the present study, we found that PFOS exposure resulted in hypergeneration of ROS in zebrafish embryos and induced phase II antioxidative enzyme activities. We showed that acute exposure of PFOS to zebrafish embryos induced Nrf2 and the MAPKs signaling pathways. In addition, by using an Nrf2 activator, we further demonstrated that Nrf2 plays a protective role against pro-oxidants to PFOS toxicity in zebrafish.

Environmental pollutants are well-known inducers of ROS, and ROS can further cause the depletion of antioxidant

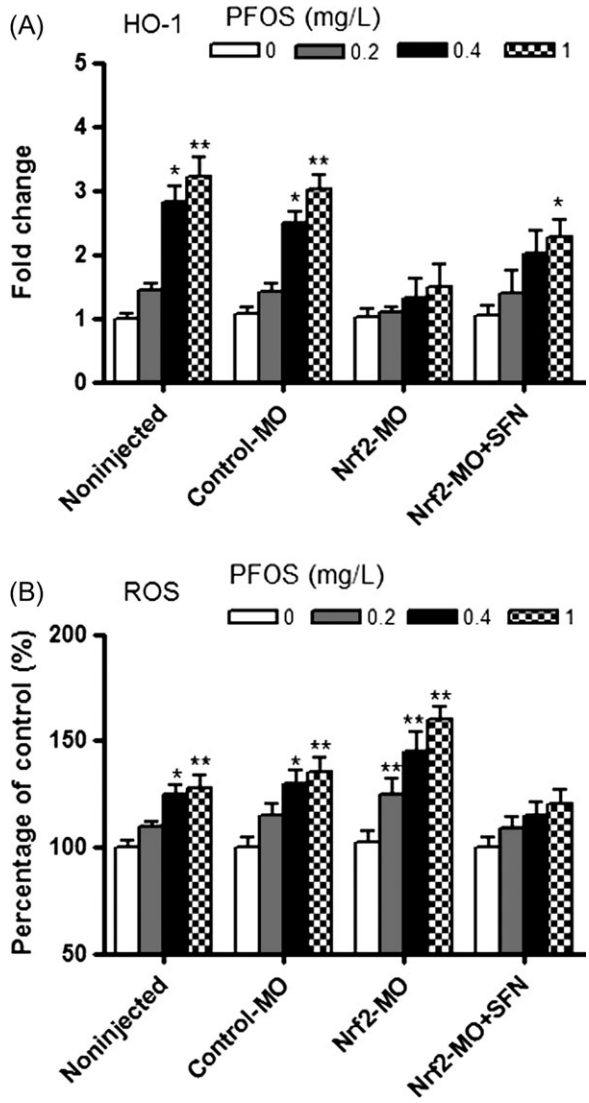

FIG. 5. Effects of Nrf2 gene knockdown with or without SFN treatment on ROS production and HO-1 induction in the zebrafish larvae upon exposure to 0, 0.2, 0.4, and 1.0 mg/l PFOS until 96 hpf: (A) real-time quantitative PCR analyses of gene expression levels of HO-1 and (B) changes in ROS formation. Values that are significantly different from the control are indicated by asterisks (one-way ANOVA, $*p < 0.05$; $**p < 0.01$). Values are presented as mean ± SEM.

defenses and mediate other oxido/reduction reactions through different metabolic pathways, such as those mediated by detoxifying enzymes (Livingstone, 2001). Our data demonstrate that PFOS is capable of inducing ROS production during early zebrafish development. It is well known that the production of ROS in fish in response to contamination is intimately associated with apoptotic cell death. Embryonic development may be especially sensitive to ROS-induced oxidative stress (Li *et al.*, 2007). We previously reported that PFOS exposure can cause cell apoptosis and alteration of certain gene expressions related to apoptosis (e.g., p53 and Bax) and result in malformation and developmental toxicity in zebrafish embryos/larvae (Shi *et al.*, 2008). Taken together, the developmental toxicity caused by PFOS exposure could be explained by induction of ROS generation and subsequent cellular apoptosis in zebrafish embryos/larvae.

Environmental stressors result in an imbalance between ROS production, and scavenging by endogenous antioxidants can directly or indirectly disturb physiological functions of many cellular macromolecules such as DNA, protein, and lipids and activate cellular stress-sensitive signaling pathways (Dröge, 2002). The involvement of antioxidative enzyme activity has been suggested to be an important role to protect cells from oxidative stress. We observed an increase in SOD, CAT and GPx activities, which were sustained in embryonic stages for zebrafish embryos exposed to PFOS. Taking into account that fish embryos contain highly unsaturated fatty acids in their lipid reservoirs, the induction of phase II enzymes contributes to the protection of embryos/larvae against lipid peroxidation in response to ROS increase. ROS and lipid peroxidation products are known to change membrane fluidity and are also involved in the intracellular signaling mechanisms, which have crucial roles in the early developmental stages (Yang *et al.*, 2003). In our previous study, we observed exposure of PFOS to zebrafish embryos significantly depressed the protein expression of annexin A4, which is an important membrane protein (Shi *et al.*, 2009). In the present study, we observed a significant increase in a lipid peroxidative product (MDA). These results suggest that PFOS exposure may affect cell membrane function and thus impair early development of zebrafish embryos.

Despite their well-known deleterious effects on biomacromolecules, ROS also functions as physiological signaling molecules (Finkel and Holbrook, 2000). In the present study, we did not investigate the mechanisms of ROS generation as our purpose was mainly focused on the ROS-induced antioxidative responses and cell signaling pathways. To elucidate the mechanisms of the pro-oxidative pathway caused by PFOS exposure in zebrafish embryos, several important genes were investigated that may be related to oxidative stress. Increasing evidences have shown that a cellular adaptive response to oxidative stress challenge is mediated by the transcription factor Nrf2 through the ARE (He *et al.*, 2007; Ho *et al.*, 2005; Pi *et al.*, 2008). The ARE is present in the promoters of genes encoding antioxidant enzymes and phase II detoxification enzymes, such as HO-1, glutathione synthetase, glutathione reductase, GPx, CAT, and SOD (Kobayashi and Yamamoto, 2006; Nguyen *et al.*, 2009). As stated above, Nfr2 is one of the major inducers of phase II detoxification enzymes and is controlled by MAPKs via several transcriptional cofactors. Previous studies have shown that Nrf2 induction is a common strategy for cells to combat chemical toxicity (He *et al.*, 2007; Ho *et al.*, 2005; Jiang *et al.*, 2009). In the present study, we observed that Nrf2 and HO-1 gene transcriptions were significantly upregulated with PFOS

exposure in zebrafish embryos. To our knowledge, this is the first report to demonstrate that PFOS activates Nrf2 through a pathway involving activation of pro-oxidative stress. In our study, we found that activation of Nrf2 by SFN could significantly reduce ROS generation induced by PFOS exposure. In addition, the mRNA expressions of p38a and p38b did not produce any statistically significant alteration in the SFN cotreatment groups. It has been reported that augmenting cellular ROS generation induces p38 MAPK activity. Thus, these results suggest that activation of the Nrf2 pathway might be a major protective response against oxidative damage in zebrafish embryos at the early developmental stages. A disruption in this pathway might affect organ toxicity caused by environmental contaminants.

HO-1 was selected for functional analysis in this study because it was previously shown to be sensitively induced in zebrafish embryos by a wide range of chemicals (Voelker *et al*., 2007, 2008). The enzyme HO-1 is involved in the response of cells to various external stimuli, such as heavy metals, cytokines, hormones, endotoxins, and heat shock (Alam and Cook, 2003). Regulation of HO-1 is very complex, and depending on the inducing agent, participation of transcription factor Nrf2, heat-shock response elements, stress response elements as well as the MAPK pathway have been reported (Alam and Cook, 2003). Gene knockdown in mammalian cells suggest a protective role of HO-1 in the case of oxidative stress (Kaizaki *et al*., 2006). Significant differences in cell viability between the control and HO-1 knockdown cells were observed in cells treated with H_2O_2. In the current study, a protective role of Nrf-2 was experimentally demonstrated for the first time in zebrafish embryos during PFOS exposure. In our experiments, we observed high levels of ROS induced by PFOS, accompanied by a significant elevation in HO-1 expression. In contrast, pretreatment with SFN increased the expression of HO-1 and significantly suppressed the level of ROS formation in zebrafish larvae. The underlying mechanism is not known but could be explained by the general cytoprotective function of HO-1 and the generation of catalytic products with antioxidant, anti-apoptotic, and cell signaling properties. HO-1 catalyses the breakdown of heme to bilirubin and its reduced form biliverdin, while both bilirubin and biliverdin exhibit antioxidant activities (Mazza *et al*., 2003) and may also protect the organism against ROS.

Activation of MAPKs pathways is known to have important roles in regulating cellular responses to oxidative stresses (Martindale and Holbrook, 2002). In the present study, we found that ERK2 and ERK3 gene expressions were unchanged, but JNK and p38 (a/b) gene expressions were significantly induced by PFOS. In general, ERK signaling pathways are related to cell survival and proliferation during oxidant injury, whereas activation of JNK and p38 are more commonly linked to apoptosis (Matsuzawa and Ichijo, 2008). The present results are in agreement with our previous study that PFOS exposure results in cell apoptosis in zebrafish larvae (Shi *et al*., 2008). Furthermore, our previous study showed that p53 and Bax, two important genes related to cell apoptosis, were both significantly upregulated upon zebrafish embryos exposure to PFOS. It has been shown that stressful stimuli can lead to activation of MAPKs, and these activated kinases can then phosphorylate and activate a number of signaling pathways, including p53 (Wu, 2004). Therefore, in light of the above results, we hypothesize that PFOS-induced apoptosis in zebrafish embryos might occur via PFOS-induced ROS generation that activates MAPK pathways; subsequently, activation of the JNK/p38 cascades induces p53 and Bax, which finally lead to apoptosis. In addition, JNK and p38 signaling pathways have important roles in embryonic development, and JNK is essential for region-specific apoptosis during early brain development in mice (Kuan *et al*., 1999). Thus, PFOS perturbs the expressions of JNK and p38, which may be associated with malformation in zebrafish embryo/larvae as observed in our previous study (Shi *et al*., 2008). Furthermore, MAPKs pathways have been reported to affect Nrf2 activity and phase II detoxifying enzymes (Limón-Pacheco *et al*., 2007). Activation of MAPKs signaling cascades could induce ARE-mediated gene expression via the Nrf2-dependent mechanism (Shen *et al*., 2004). These results suggest that MAPKs pathways may also contribute to cellular defenses against oxidative stress in zebrafish embryo exposed to PFOS.

To evaluate the role of MAPK pathways in the regulation of HO-1 responses, we used MAPK inhibitors to block $p38^{MAPK}$, ERK, and JNK pathways with or without SFN treatment. The results showed that inhibition of the MAPK pathways alone did not change PFOS-induced HO-1 gene expression and ROS production. Although the MAPK pathway is activated by PFOS exposure, MAPK activation did not seem to participate in the induction of HO-1 since the addition of MAPK inhibitors did not affect HO-1 mRNA levels. A recent study also reported that inhibition of ERK did not affect HO-1 gene expression in rat pheochromocytoma PC12 cells exposed to kaempferol and rhamnocitrin (Hong *et al*., 2009). On the other hand, cotreatment of SFN with MAPK inhibitors further upregulated HO-1 gene expression. SFN is a very potent activator of the transcription factor Nrf2 and regulates phase II enzymes, including HO-1, which plays a critical role in metabolism and excretion of xenobiotics (Fahey and Talalay, 1999). It therefore seems likely that HO-1 induction confers antioxidant protection. In the present study, we demonstrated that activation of Nrf2 by SFN treatment effectively protected against PFOS-induced ROS generation.

In order to further explore the possible role of Nrf2 in protection against PFOS-induced oxidative stress, prior to PFOS treatment, the Nrf2 gene was knocked down in zebrafish embryos. Our data showed that PFOS induced a marked increase in HO-1 levels, and knockdown of Nrf2 markedly attenuated this increase. Our studies thus demonstrated that Nrf2 is one of the essential regulators of PFOS-induced HO-1 expression. Although there are no reports regarding PFCs

induced oxidative stress related to activation of Nrf2, many studies have shown the role of Nrf2 in antioxidative stress with other toxicants. For instance, a recent study demonstrated that Nrf2 knockdown prevented upregulation of other antioxidative genes (e.g., SOD and GPx) with exposure to polycyclic aromatic hydrocarbons (PAHs) in zebrafish embryos (Timme-Laragy *et al*., 2009), suggesting that Nrf2 is an important response element in these promoters. The importance of Nrf2 in embryonic development has also been demonstrated. For example, Nrf2 knockout mice have impaired oxidative stress defense mechanisms (Leung *et al*., 2003), and knockdown of Nrf2 increased mortality following PAH exposure to zebrafish embryos (Timme-Laragy *et al*., 2009). Knockdown of Nrf2 gene also significantly increased the ROS generation and apoptotic cell death in cadmium-treated rat kidney cells (Chen and Shaikh, 2009). Based on these observations, it seems reasonable to conclude that Nrf2 activation confers protection against toxicant-induced oxidative stress.

In summary, the present study demonstrates that zebrafish embryos/larvae can be used to study the function of a single gene with respect to oxidative toxicity. PFOS exposure caused hypergeneration of ROS in zebrafish embryos, which in turn induced phase II detoxification enzymes to protect oxidative damage. The gene expression patterns of Nrf2 and HO-1 suggested that a protective role of the Nrf2 pathway against oxidative stress exists *in vivo*. The JNK and p38 pathways may also be involved in PFOS-induced cell apoptosis in zebrafish embryos at early developmental stages. However, the relationship between the protective role of certain gene functions and developmental toxicity remains to be further investigated.

FUNDING

The National Nature Science Foundation of China (20890113, 20877094); the State Key Laboratory of Freshwater Ecology and Biotechnology (2008FBZ10).

ACKNOWLEDGMENTS

We thank Dr Zhan Yin for technical assistance with gene knockdown.

REFERENCES

Alam, J., and Cook, J. L. (2003). Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. *Curr. Pharm. Des.* **9,** 2499–2511.

Ankley, G. T., Kuehl, D. W., Kahl, M. D., Jensen, K. M., Linnum, A., Leino, R. L., and Villeneuvet, D. A. (2005). Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **24,** 2316–2324.

Chen, J., and Shaikh, Z. A. (2009). Activation of Nrf2 by cadmium and its role in protection against cadmium-induced apoptosis in rat kidney cells. *Toxicol. Appl. Pharmacol.* **241,** 81–89.

Copple, I. M., Goldring, C. E., Kitteringham, N. R., and Park, B. K. (2008). The Nrf2-Keap1 defence pathway: role in protection against drug-induced toxicity. *Toxicology* **246,** 24–33.

Deng, J., Yu, L., Liu, C., Yu, K., Shi, X., Yeung, L. W., Lam, P. K., Wu, R. S., and Zhou, B. (2009). Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. *Aquat. Toxicol.* **93,** 29–36.

Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiol. Rev.* **82,** 47–95.

Fahey, J. W., and Talalay, P. (1999). Antioxidant functions of sulforaphane: a potent inducer of phase II detoxication enzymes. *Food Chem. Toxicol.* **37,** 973–979.

Finkel, T., and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408,** 239–247.

Flohé, L., and Otting, F. (1984). Superoxide dismutase assays. *Methods Enzymol.* **105,** 93–104.

Fujii, R., Yamashita, S., Hibi, M., and Hirano, T. (2000). Asymmetric p38 activation in zebrafish: its possible role in symmetric and synchronous cleavage. *J. Cell Biol.* **150,** 1335–1348.

Giesy, J. P., and Kannan, K. (2001). Global distribution of perfluorooctane sulfonate in wildlife. *Environ. Sci. Technol.* **35,** 1339–1342.

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase. *Anal. Biochem.* **106,** 207–221.

Hagenaars, A., Knapen, D., Meyer, I. J., van der Ven, K., Hoff, P., and De Coen, W. (2008). Toxicity evaluation of perfluorooctane sulfonate (PFOS) in the liver of common carp (*Cyprinus carpio*). *Aquat. Toxicol.* **88,** 155–163.

He, X., Lin, G. X., Chen, M. G., Zhang, J. X., and Ma, Q. (2007). Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the nucleus and disrupting the nuclear Nrf2/Keap1 association. *Toxicol. Sci.* **98,** 298–309.

Ho, H. K., White, C. C., Fernandez, C., Fausto, N., Kavanagh, T. J., Nelson, S. D., and Bruschi, S. A. (2005). Nrf2 activation involves an oxidative-stress independent pathway in tetrafluoroethylcysteine-induced cytotoxicity. *Toxicol. Sci.* **86,** 354–364.

Hong, J., Yen, J., Wang, L., Lo, Y., Chen, Z., and Wu, M. (2009). Regulation of heme oxygenase-1 expression and MAPK pathways in response to kaempferol and rhamnocitrin in PC12 cells. *Toxicol. Appl. Pharmacol.* **237,** 59–68.

Hsieh, C., Jeng, S., Hsieh, M., Chen, Y., Rau, C., Lu, T., and Chen, S. (2008). Statin-induced heme oxygenase-1 increases NF-{kappa}B activation and oxygen radical production in cultured neuronal cells exposed to lipopolysaccharide. *Toxicol. Sci.* **102,** 150–159.

Huang, T. S., Olsvik, P. A., Krovel, A., Tung, H. S., and Torstensen, B. E. (2009). Stress-induced expression of protein disulfide isomerase associated 3 (PDIA3) in Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol.* **154B,** 435–442.

Jiang, T., Huang, Z., Chan, J., and Zhang, D. (2009). Nrf2 protects against As(III)-induced damage in mouse liver and bladder. *Toxicol. Appl. Pharmacol* **240,** 8–14.

Johnson, G. L., and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298,** 1911–1912.

Kaizaki, A., Tanaka, S., Ishige, K., Numazawa, S., and Yoshida, T. (2006). The neuroprotective effect of heme oxygenase (HO) on oxidative stress in HO-1 siRNA-transfected HT22 cells. *Brain Res.* **1108,** 39–44.

Kannan, K., Tao, L., Sinclair, E., Pastva, S. D., Jude, D. J., and Giesy, J. P. (2005). Perfluorinated compounds in aquatic organisms at various trophic levels in a Great Lakes food chain. *Arch. Environ. Contam. Toxicol.* **48,** 559–566.

Kensler, T. W., Wakabayashi, N., and Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* **47,** 89–116.

Kobayashi, M., Li, L., Iwamoto, N., Nakajima-Takagi, Y., Kaneko, H., Nakayama, Y., Eguchi, M., Wada, Y., Kumagai, Y., and Yamamoto, M. (2009). The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Mol. Cell. Biol.* **29,** 493–502.

Kobayashi, M., and Yamamoto, M. (2006). Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.* **46,** 113–140.

Krens, S. F., He, S., Spaink, H. P., and Snaar-Jagalska, B. E. (2006). Characterization and expression patterns of the MAPK family in zebrafish. *Gene Expr. Patterns* **6,** 1019–1026.

Krøvel, A. V., Softeland, L., Torstensen, B., and Olsvik, P. A. (2008). Transcriptional effects of PFOS in isolated hepatocytes from Atlantic salmon *Salmo salar* L. *Comp. Biochem. Physiol.* **148C,** 14–22.

Kuan, C. Y., Yang, D. D., Samanta Roy, D. R., Davis, R. J., Rakic, P., and Flavell, R. A. (1999). The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22,** 667–676.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., and Seed, J. (2007). Perfluoroalkyl acids: a review of monitoring and toxicological findings. *Toxicol. Sci.* **99,** 366–394.

Leung, L., Kwong, M., Hou, S., Lee, C., and Chan, J. Y. (2003). Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. *J. Biol. Chem.* **278,** 48021–48029.

Li, S. Y., Sigmon, V. K., Babcock, S. A., and Ren, J. (2007). Advanced glycation endproduct induces ROS accumulation, apoptosis, MAP kinase activation and nuclear O-GlcNAcylation in human cardiac myocytes. *Life Sci.* **80,** 1051–1056.

Limón-Pacheco, J. H., Hernandez, N. A., Fanjul-Moles, M. L., and Gonsebatt, M. E. (2007). Glutathione depletion activates mitogen-activated protein kinase (MAPK) pathways that display organ-specific responses and brain protection in mice. *Free Radic. Biol. Med.* **43,** 1335–1347.

Liu, C., Yu, K., Shi, X., Wang, J., Lam, P. K., Wu, R. S., and Zhou, B. (2007a). Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia (*Oreochromis niloticus*). *Aquat. Toxicol.* **82,** 135–143.

Liu, S. I., Huang, C. C., Huang, C. J., Wang, B. W., Chang, P. M., Fang, Y. C., Chen, W. C., Wang, J. L., Lu, Y. C., Chu, S. T., *et al.* (2007b). Thimerosal-induced apoptosis in human SCM1 gastric cancer cells: activation of p38 MAP kinase and caspase-3 pathways without involvement of $[Ca^{2+}]_i$ elevation. *Toxicol. Sci.* **100,** 109–117.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25,** 402–408.

Livingstone, D. R. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar. Pollut. Bull.* **42,** 656–666.

Luck, H. (1963). Catalase. In *Methods of Enzymatic Analysis* (H.-U. Bergmeyer, Ed.), pp. 885–888. Academic Press, New York.

Martindale, J. L., and Holbrook, N. J. (2002). Cellular response to oxidative stress: signaling for suicide and survival. *J. Cell Physiol.* **192,** 1–15.

Matsuzawa, A., and Ichijo, H. (2008). Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochim. Biophys. Acta.* **1780,** 1325–1336.

Mazza, F., Goodman, A., Lombardo, G., Vanella, A., and Abraham, N. G. (2003). Heme oxygenase-1 gene expression attenuates angiotensin II-mediated DNA damage in endothelial cells. *Exp. Biol. Med.* **228,** 576–583.

Nguyen, T., Nioi, P., and Pickett, C. B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J. Biol. Chem.* **284,** 13291–13295.

Niture, S. K., Kaspar, J. W., Shen, J., and Jaiswal, A. K. (2009). Nrf2 signaling and cell survival. *Toxicol. Appl. Pharmacol.* **244,** 37–42.

Oakes, K. D., Sibley, P. K., Martin, J. W., MacLean, D. D., Solomon, K. R., Mabury, S. A., and Van Der Kraak, G. J. (2005). Short-term exposures of fish to perfluorooctane sulfonate: acute effects on fatty acyl-CoA oxidase activity, oxidative stress, and circulating sex steroids. *Environ. Toxicol. Chem.* **24,** 1172–1181.

Ohkawa, H., Ohishi, N., and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95,** 351–358.

Pi, J., Zhang, Q., Woods, C. G., Wong, V., Collins, S., and Andersen, M. E. (2008). Activation of Nrf2-mediated oxidative stress response in macrophages by hypochlorous acid. *Toxicol. Appl. Pharmacol.* **226,** 236–243.

Sen, C. K., and Packer, L. (1996). Antioxidant and redox regulation of gene transcription. *FASEB J.* **10,** 709–720.

Shen, G., Hebbar, V., Nair, S., Xu, C., Li, W., Lin, W., Keum, Y.-S., Han, J., Gallo, M. A., and Kong, A. N. T. (2004). Regulation of Nrf2 transactivation domain activity. *J. Biol. Chem.* **279,** 23052–23060.

Shi, X., Du, Y., Lam, P. K. S., Wu, R. S. S., and Zhou, B. (2008). Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. *Toxicol. Appl. Pharmacol.* **230,** 23–32.

Shi, X., Yeung, L. W., Lam, P. K. S., Wu, R. S. S., and Zhou, B. (2009). Protein profiles in zebrafish (*Danio rerio*) embryos exposed to perfluorooctane sulfonate. *Toxicol. Sci.* **110,** 334–340.

Timme-Laragy, A. R., Van Tiem, L. A., and Linney, E. A. (2009). Di Giulio, R. T. (2009). Antioxidant responses and NRF2 in synergistic developmental toxicity of PAHs in zebrafish. *Toxicol. Sci.* **109,** 217–227.

Voelker, D., Stetefeld, N., Schirmer, K., and Scholz, S. (2008). The role of cyp1a and heme oxygenase 1 gene expression for the toxicity of 3,4-dichloroaniline in zebrafish (*Danio rerio*) embryos. *Aquat. Toxicol.* **86,** 112–120.

Voelker, D., Vess, C., Tillmann, M., Nagel, R., Otto, G. W., Geisler, R., Schirmer, K., and Scholz, S. (2007). Differential gene expression as a toxicant-sensitive endpoint in zebrafish embryos and larvae. *Aquat. Toxicol.* **81,** 355–364.

Wei, Y., Shi, X., Zhang, H., Wang, J., Zhou, B., and Dai, J. (2009). Combined effects of polyfluorinated and perfluorinated compounds on primary cultured hepatocytes from rare minnow (*Gobiocypris rarus*) using toxicogenomic analysis. *Aquat. Toxicol.* **95,** 27–36.

Wu, G. S. (2004). The functional interactions between the p53 and MAPK signaling pathways. *Cancer Biol. Ther.* **3,** 156–161.

Yamashita, M. (2003). Apoptosis in zebrafish development. *Comp. Biochem. Physiol.* **136B,** 731–742.

Yang, Y., Sharma, R., Sharma, A., Awasthi, S., and Awasthi, Y. C. (2003). Lipid peroxidation and cell cycle signaling: 4-hydroxynonenal, a key molecule in stress mediated signaling. *Acta. Biochim. Pol.* **50,** 319–336.

Zipper, L. M., and Mulcahy, R. T. (2000). Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. *Biochem. Biophys. Res. Commun.* **278,** 484–492.