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Characterization of phospholipid hydroperoxide glutathione metabolizing peroxidase (*gpx4*) isoforms in Coho salmon olfactory and liver tissues and their modulation by cadmium

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Abstract

Exposure to environmental contaminants, including various pesticides and trace metals, can disrupt critical olfactory-driven behaviors of fish such as homing to natal streams, mate selection, and an ability to detect predators and prey. These neurobehavioral injuries have been linked to reduced survival, and population declines. Despite the importance of maintaining proper olfactory signaling processes in the presence of chemical exposures, little is known regarding chemical detoxification in the salmon olfactory system, and in particular, the antioxidant defenses that maintain olfactory function. An understudied, yet critical component of cellular antioxidant defense is phospholipid hydroperoxide glutathione peroxidase (PHGPx/GPx4), an isoform within the family of selenium-dependent glutathione peroxidase (GPx) enzymes that can directly reduce lipid peroxides and other membrane-bound complex hydroperoxides. In this study, we cloned two *gpx4* isoforms (*gpx4a* and *gpx4b*) from Coho salmon olfactory tissues and compared their modulation in olfactory and liver tissues by cadmium, an environmental pollutant and olfactory toxicant that cause oxidative damage as a mechanism of toxicity. Amino acid sequence comparisons of the two *gpx4* isoforms shared 71% identity, and also relatively high sequence identities when compared with other fish GPx4 isoforms. Sequence comparisons with human GPx4 indicated conservation of three important active-sites at selenocysteine (U46), glutamine (Q81), and tryptophan (W136), suggesting similar catalytic activity between fish and mammalian GPx4 isoforms. Tissue profiling confirmed the expression of *gpx4a* and *gpx4b* in all ten Coho tissues examined. The expression of *gpx4* mRNAs in the Coho olfactory system was accompanied by comparably high initial rates of GPx4 enzymatic activity in mitochondrial and cytosolic fractions. Exposure to low (3.7 ppb) and high (347 ppb) environmental Cd concentrations for 24–48 hrs significantly decreased *gpx4a* expression in Coho olfactory rosettes, whereas olfactory *gpx4b* mRNA expression was not modulated by exposures at these concentrations. In summary, Coho salmon express two paralogs of *gpx4*, a key enzyme in the maintenance of signal transduction processes that protect against cellular oxidative damage. The Cd-associated downregulation of salmon olfactory *gpx4a* expression in particular, may be associated with the loss of olfactory signal transduction that accompanies metal-associated loss of olfaction in salmonids.

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Keywords

GPx4; cadmium; olfactory injury; Coho salmon

1. Introduction

The decline of Pacific salmon populations in the Western United States has been associated with the loss of habitat quality and contamination of surface waters and sediments (reviewed in (Lackey, 2003)). Environmental pollutants, including trace metals and pesticides are a particular concern due to their neurotoxic effects on the salmon olfactory system (Baatrup, 1991; Baldwin et al., 2003; Jarrard et al., 2004; Scott et al., 2003; Tierney et al., 2006). The olfactory rosettes constitute the major component of the salmon peripheral olfactory system and are in direct contact with the surrounding water, which renders this system vulnerable to the toxicity of dissolved pollutants. Damage to olfactory sensory neurons (OSNs) located within the olfactory epithelium (OE) (Laberge and Hara, 2001) leads to impaired olfactory function, resulting in the loss of critical behaviors such as normal swimming, detection of predators and prey, recognition of reproductive chemical cues, and homing to natal streams, which ultimately affects species survival (Laetz et al., 2009; Palm and Powell, 2010; Sandahl et al., 2007).

Despite their critical function in mediating behavioral processes that can affect salmon survival, little is known regarding the chemical biotransformation capacity of the salmon olfactory system. We have shown that the peripheral olfactory system of Coho salmon expresses a number of glutathione *S*-transferases (GSTs), including several isoforms that detoxify secondary products of oxidative damage (Espinoza et al., 2011). In addition to GSTs, the glutathione peroxidases (GPx) are another important family of glutathione-associated enzymes that participate in cellular defense via enzymatic reduction of reactive cellular hydroperoxides in the presence of glutathione (GSH) (Conrad et al., 2005; Imai and Nakagawa, 2003; Kuhn and Borchert, 2002). Of these enzymes, the phospholipid hydroperoxide metabolizing glutathione peroxidase (PHGPx/GPx4) represents a unique member of the tetrameric selenium-contained GPx family (Savaskan et al., 2007b; Ufer and Wang, 2011). GPx4 possesses a distinctive monomeric structure and hydrophobic surface (Ursini et al., 1985), which underlies a unique catalytic activity capable of reducing complex membrane-bound hydroperoxides and lipid peroxides, such as phospholipid and cholesterol hydroperoxides (Roveri et al., 1994; Schnurr et al., 1996; Thomas et al., 1990). Since its initial purification from pig liver (Ursini et al., 1982), GPx4 has been extensively studied in mammalian models and its function addressed through several genetic approaches. For example, *GPx4*$^{+/-}$ knockout mice have an increased sensitivity to oxidative stress (Imai et al., 2003; Yant et al., 2003), whereas mice that overexpress *GPx4* are protected from liver oxidative damage (Ran et al., 2004), and this effect is also observed in cortical neurons (Ran et al., 2006).

GPx4 isoforms have been identified in several fish species, including common carp (*Cyprinus carpio*, *gpx4a* and *gpx4b*) (Hermesz and Ferencz, 2009), zebrafish (*Danio rerio*, *gpx4a* and *gpx4b*) (Kryukov and Gladyshev, 2000), Atlantic salmon (*Salmo salar, gpx4b*) and southern bluefin tuna (*Thunnus maccoyii*, *gpx4*) (Thompson et al., 2010). However, little is known about how these unique GSH-dependent enzymes respond to environmental stressors or if they are functional in the fish olfactory system. The goal of the present study was to characterize *gpx4* expression and distribution in Coho salmon (*Oncorhynchus kisutch*), with a particular emphasis on function in the salmon olfactory system. A secondary goal of the study was to examine *gpx4* modulation in salmon olfactory tissues on exposure to a prototypical toxicant that causes oxidative stress. In this regard, cadmium (Cd) is a

ubiquitously distributed trace metal that is toxic to liver and kidney tissues and also disrupts normal behavioral and alarm responses at environmental exposure levels (Scott et al., 2003; Stromberg et al., 1983). These neurobehavioral injuries are associated with the accumulation of Cd in the olfactory system and disruption of normal olfactory function (Gottofrey and Tjälve, 1991; Tjälve et al., 1986; Tjälve and Henriksson, 1999). Studies in numerous species, including fish, have demonstrated that a major mechanism of Cd toxicity involves enhancing oxidative stress via increased production of cellular reactive oxygen species (ROS) (Hart et al., 1999; Risso-de Faverney et al., 2004; Thevenod et al., 2000; Wang et al., 2004; Watanabe and Suzuki, 2002). After characterizing the presence and functionality of the *gpx4* paralogs, we challenged Coho with environmentally-relevant levels of Cd associated with modulation of cell signaling and also oxidative stress to determine such effects on *gpx4* mRNA expression.

2. Material and methods

2.1 Animals and tissue processing

Juvenile Coho salmon (~1 year of age) and adult Coho (~2 year old) were provided by the National Oceanic and Atmospheric Administration (NOAA), Seattle, Washington. Fish were raised in cylindrical tanks with recirculated freshwater under natural photoperiod in dechlorinated municipal water. Fish were fed commercial dry food pellets (BioOregon, Warrenton, OR, USA) once a day. Water quality conditions were typically ~120 mg/L as $CaCO_3$, pH 6.6, 10–12 °C, and 8.1 mg/L dissolved oxygen content. Twenty-four hr prior to initiation of exposures, the juvenile Coho were transferred to 120 L aquaria receiving filtered city water (pH=7.1 ± 0.1, 12 ± 1 °C, alkalinity 80 mg/L, hardness 50 mg/L, dissolved oxygen 8.6 ± 0.58 mg/L). Following the exposures, fish were anesthetized with tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA) prior to cervical dislocation. For tissue expression analysis, gills, liver, olfactory rosettes, kidney, spleen, ovary or testis, heart, brain and pyloric caeca were collected from eight adult Coho salmon, rinsed in 1X PBS (pH=7.0), placed in 1 ml Trizol (Invitrogen, Carlsbad, CA) and snap-frozen in liquid nitrogen. For enzyme activity analysis, liver and olfactory rosettes were collected from 20 adult Coho salmon, snap-frozen in liquid nitrogen, and store stored at −80 °C until processing.

2.2 Cloning of *gpx4* isoforms by RACE

Total RNA was isolated from Coho salmon tissues using the Trizol method according to manufacturer's instructions. RNA concentrations and purity were verified on a ND100 nanodrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA). First strand cDNA was synthesized from 1 μg of total RNA using oligo (dT) primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad CA.), and 1 μL of cDNA was used as templates for all PCR amplifications. All amplified products were cloned into a TOPO cloning vector (Invitrogen) and sequenced at the University of Washington, Department of Biochemistry Sequencing Facility. Rapid amplification of cDNA ends (RACE-PCR) using the SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA) together with MMLV Reverse Transcriptase was used to amplify *gpx4*, according to the supplier's protocol. Coho liver cDNA was used to generate all RACE products. Gene-specific RACE primers (primers 1 and 2, Table 1) for *gpx4b* were designed against *Salmo salar gpx4b* partial sequence (GenBank accession no. **BT044014**). Full-length *gpx4b* was cloned from olfactory and liver cDNA libraries with primer 3 set and sequenced in both directions.

A degenerate primer (primer 4, Table 1) for *gpx4a* 3′RACE was designed by identifying highly conserved regions of *gpx4a* sequences from three other fish species, including zebrafish (GenBank accession no. **NM001007282**); carp (GenBank accession no.

FJ656211); southern bluefin tuna (GenBank accession no. **EF452498**), and by omitting conserved regions present in *gpx4b*. For 5′RACE, a PCR primer was designed (primer 5) from a 3′RACE PCR product. Full-length *gpx4a* was cloned from olfactory and liver cDNA libraries with primer set 6 (Table 1) and sequenced. Sequence homology searches were carried out using BLAST (Basic Local Alignment Search Tool), whereas sequence alignments were performed using ClustalW (San Diego Biology Workbench 3.2, http://workbench.sdsc.edu/). The final contiguous sequences were compiled using DNASTAR Lasergene8 sequence analysis software (DNASTAR, Madison, WI).

2.3 *Gpx4a* and *gpx4b* tissue-specific mRNA expression

Primers for quantitative real-time PCR analysis of *gpx4* isoforms in salmon tissues were designed using Primer 3 software program (San Diego Biology Workbench 3.2, http://workbench.sdsc.edu/). Each primer pair produced a single PCR product as evidenced by melt curve analysis and gel electrophoresis (primers 7–11, Table 1). After PCR product validation and assay optimization, qPCR assays were conducted and analyzed using the relative standard curve method (Espinoza et al., 2011). Standards for quantification were created from gel-purified PCR products using QIAX II kit (Qiagen Inc.) and quantified before serial dilutions from 100 to 0.001 pg. All standard curves were generated revealed high coefficients of determination ($R^2 > 0.99$). DNA SYBR Green master mix (Finnzymes), 0.3 μM of each primer, and molecular grade water were used to achieve the final reaction volume of 20 uL for each PCR triplicate. PCR amplifications were performed using a Bio-Rad IQ5 thermocycler (Hercules, CA) for 40 cycles with denaturation at 94 °C for 10 s, annealing at optimum temperature for primers (55–58 °C) for 20 s, and extension at 72 °C for 12 s. For quality control purposes, no template controls, as well as melt curve analyses were completed for all reactions. For *gpx4* tissue profiling, gene expression quantities were normalized against β-actin, and ratios were calculated for the comparison between two *gpx4* isoforms.

2.4 Analysis of GPx4 catalytic activities

To verify the presence of functional GPx4-associated catalytic activity, liver tissues from three juvenile Coho were thawed and rinsed in ice-cold 0.9% KCl buffer to remove the blood. Olfactory rosettes were pooled from a total of six individual Coho to provide enough tissue for n=3 pools. Tissue samples were homogenized with Potter–Elvehjem Teflon tissue homogenizer in three volumes of homogenization buffer (250 mM sucrose, 10 mM tris, 1 mM EDTA, 0.2 mM DTT, 0.1 mM PMSF, pH=7.4) at 0–4°C. The homogenate was centrifuged at 600 *g* for 15 min to discard cell debris and the nuclear fraction. The supernatant fluid was centrifuged at 10,000 *g* for 30 min to isolate the bulk of mitochondria, and the resulting supernatant fluid was centrifuged at 13,000 *g* for 20 min. The mitochondrial fractions were prepared by washing in homogenization buffer, re-centrifuging, and resuspending in homogenization buffer. The 13,000 *g* supernatants were centrifuged at 105,000 *g* for 1 h to obtain the cytosolic fractions. All cellular fractions were stored at −80 °C until use.

Whereas all GPx isozymes can catalyze the reduction of CuOOH, only GPx4 isoforms have the ability to reduce phosphatidylcholine hydroperoxide (PCOOH) with GSH, and thus this substrate can reflect GPx4-specific activity. PCOOH was prepared by oxidation of 1,2-dilinoleoyl-3-phosphatidylcholine (PC) with soybean lipoxidase as described previously (Garry et al., 2008). The concentration of PCOOH was determined spectrophotometrically using an extinction coefficient of 25,000 M^{-1} cm^{-1} at 234 nm. GPx4 catalytic activity was determined spectrophotometrically by a coupled enzyme assay (Maiorino et al., 1990; Weitzel et al., 1990). Briefly, 10 uL of GPx4-containing sample were added to a total volume of 1.0 mL buffer mixture containing 0.1 M Tris-HCl (pH=7.6), 5 mM EDTA, 1 mM

sodium azide, 3 mM GSH, 0.2 mM NADPH, 0.1% peroxide free Triton X-100 (AMRESCO, Solon, OH), 1.2 U glutathione reductase (Sigma, specific activity 230 U/mg protein). The reaction mixture was pre-incubated for 3 min at room temperature and the initial slope was recorded as non-specific NADPH and glutathione oxidation. The reaction was then initiated by addition of substrate (4.2–5.9 nmol PCOOH, or 100 nmol CuOOH), and the oxidation of NADPH was monitored at ΔA_{340}nm for 3 min. Negative controls were conducted in parallel in the absence of enzyme, and the resulting GPx4 activity was calculated from the difference between the two slopes using an extinction coefficient of 6220 M^{-1} cm^{-1}. Protein concentrations were determined using the Bradford method (Bradford, 1976).

2.5 Effect of *in vivo* Cd exposures on *gpx4* mRNA expression in Coho liver and olfactory rosettes

To analyze the effects of Cd on Coho liver and olfactory *gpx4* mRNA expression, 8 juvenile Coho salmon (15.0 g ± 5.7) per treatment group were exposed to the intended concentrations of 0, 3.1 and 310 ppb cadmium ($CdCl_2$, Mallinckrodt Baker, Phillipsburg, NJ) in 120 L aquaria contained within a large chilled re-circulating water bath and aerated by individual air stones for 24 and 48 hrs. The exposures were accomplished using a 90% renewal with water containing test agent at 24 hours. Water samples were taken pre- and post-exposures to assess the nominal concentrations (Frontier GeoSciences Inc. Seattle WA) and the Cd concentrations reported herein reflect measured waterborne levels (Espinoza et al., 2011). Specifically, the actual measured waterborne cadmium concentrations in the tanks at the initiation of exposures were 3.7 and 347 ppb in the low and high dose exposure tanks, respectively. Hereafter, measured cadmium concentrations are used in all text, tables and figures. Following exposures, the olfactory rosettes and liver tissues were collected from n=8 anesthetized fish per treatment as described above. Analysis of olfactory β-actin mRNA expression showed unacceptable variation among the treatments, thus 3 internal reference genes (β-actin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L9 (rpL9)) were used to create geometric mean values for normalization of the *gpx4* mRNA expression. Normalization factors were determined using geNorm (version 3.5) software (http://medgen.ugent.be/~jvdesomp/genorm/).

2.6 Statistical Analysis

Prior to statistical analyses, all data were inspected for homogeneity of variances using the Bartlett's test. In some cases, such as tissue specific profile data, the distributions did not follow parametric distributions. Accordingly, tissue differences among *gpx4a* and *gpx4b* were assessed by using the (Wilcoxon) Mann-Whitney *U* test. To analyze the effects of Cd exposures on *gpx4* mRNA expression, data sets conforming to normal distributions were assessed for significance using a one-way ANOVA followed by a Dunnett's test. For non-parametric distributions, data were assessed for significance using Kruskal-Wallis nonparametric one-way analysis of variance test followed by a Dunn's test. All data were considered statistically significant at $p \leq 0.05$. All statistical analyses were conducted using GraphPad Prism Ver 5.0 (Graph Pad Software Inc, San Diego, CA, USA).

3. Results

3.1 Cloning of *gpx4a* and *gpx4b* isoforms

Full-length *gpx4a* and *gpx4b* cDNAs were successfully cloned from Coho salmon olfactory and liver total RNA by RACE-PCR. The full length *gpx4a* cDNA contained a 5′-UTR of 31 bp, an open reading frame (ORF) of 603 bp and also a 3′-UTR of 333 bp (GenBank accession no. **JN967674**). Inspection of the *gpx4a* ORF revealed two potential protein translation start sites (5′-ATG and 3′-ATG) encoding deduced proteins of 170 and 200 amino acids in length. The *gpx4b* cDNA contained a 5′-UTR of 62 bp, an open reading

frame (ORF) of 576 bp, and a 3′-UTR of 436 bp (GenBank accession no. **JN967675**). The ORF has two possible protein translation start sites encoding deduced proteins of 170 and 191 amino acids in length (Figure 1A). Amino acid sequence comparisons of the two isoforms revealed 71% identities. The phylogenetic analysis of the two isoforms revealed high sequence identities when compared with other fish and higher vertebrate GPx4 isoforms (Figure 1B). For example, Coho GPx4a shared 83% identity with carp GPx4a, and Coho GPx4b shared 81% identity with carp GPx4b. Both Coho GPx4 isoforms shared ~60% identity with human GPx4. Similar to mammalian GPx4, the predicted Coho GPx4 protein showed a potential mitochondrial targeting sequence (30 amino acids in length for GPx4a and 21 amino acids in length for GPx4b, respectively, Figure 1A).

3.2 Tissue expression profiles of *gpx4* genes

Expression of the *gpx4a* and *gpx4b* were analyzed in 10 tissues using qRT-PCR. In these experiments, β-actin mRNA expression levels did not differ among the tissues and was consequently used as the internal reference gene for normalization purposes. The highest *gpx4a* mRNA expression was found in pyloric caeca, followed by brain, heart and liver (Figure 2). By contrast, *gpx4b* showed highest mRNA expression in ovary, followed by pyloric caeca, heart, liver and brain (Figure 2). In general, *gpx4b* mRNA expression exceeded that of *gpx4a* in all tissues with the exception of the pyloric caeca. The ratio of *gpx4b*/*gpx4a* was highest (−39) in ovary, and lowest in pyloric caeca (−0.7). With the exception of the gonads, no sex differences in constitutive gene expression were observed.

3.4 GPx catalytic enzyme activities

The results of the total GPx and GPx4-specific catalytic activity analyses in adult Coho liver and olfactory rosette tissues are shown in Table 2. As observed, the mitochondrial and cytosolic GPx4 isoforms were distinguished from total tissue GPx by using the substrate PCOOH, a specific substrate of GPx4. As observed, the total tissue initial rate GPx activities were approximately 2- fold higher in olfactory mitochondrial and cytosolic fractions compared to those in the corresponding liver subcellular fractions (Table 2). Further, higher GPx activities were observed in cytosolic fractions than mitochondrial fractions in both the olfactory and liver tissues (Table 2). The presence of GPx4-specific catalytic activity accompanied *gpx4* mRNA expressions in olfactory and liver subcellular fractions, and did not markedly differ among the two tissues.

3.5 Effect of Cd on olfactory and liver *gpx4* mRNA expression

As observed in Figure 3, *gpx4* mRNA expressions in olfactory and liver tissues were modulated by exposure to both doses of Cd. In olfactory rosettes, exposure to 347 ppb Cd significantly decreased *gpx4a* expression (48% loss by 24hr; 44% loss by 48 hr exposure, Figure 3A). By contrast, olfactory *gpx4b* mRNA expression was not extensively modulated by Cd. A transient effect of Cd in the expression of liver *gpx4* was also observed (Figure 3B). By 24 hr, exposure to the low dose of Cd caused mild changes of *gpx4a* and *gpx4b* expression (13% and 18% loss, respectively). As similarly observed in olfactory tissues, the higher Cd dose elicited a more extensive effect on liver *gpx4a* and *gpx4b* expression (40% and 37% loss by 24 hr, respectively). Longer exposure periods caused minor (20–22%) losses of liver *gpx4a* and *gpx4b* expression by the low Cd dose, whereas at higher Cd exposures, a 33% loss in *gpx4b* expression was observed (Figure 3).

4. Discussion

Our recent study showing the expression of eight *GST* isoforms in Coho olfactory tissues, including isoforms that detoxify secondary byproducts of oxidative stress, suggests that the cellular antioxidant pathways that maintain redox status and protect against cellular

oxidative damage may be important in maintaining olfactory processes (Espinoza et al., 2011). Among GSH-dependent antioxidant enzymes, the unique substrate profile of cellular GPx4 is of particular importance in maintaining olfactory signal transduction profiles by nature of their functional ability to modulate signal transduction, as well as reduce potentially toxic intracellular lipid peroxides (Ursini et al., 1985; Ursini et al., 1982). Our demonstration of two *gpx4* paralogs in Coho olfactory tissues, accompanied by comparably high GPx4 catalytic activities, is consistent with an active GSH-associated olfactory antioxidant defense system, with a function in maintaining normal olfactory signal transduction in the face of environmental stress.

Studies in other species indicate a substantial role for GPx4 in maintaining neuronal cell function, especially under conditions of oxidative stress (Ran et al., 2006; Savaskan et al., 2007a; Savaskan et al., 2007b; Seiler et al., 2008). Furthermore, GPx4 is highly expressed in several regions of the rodent brain (i.e. cerebral cortex, hippocampus) and olfactory tissues, including olfactory epithelium and olfactory bulb (Schneider et al., 2006; Zhang et al., 2008), supporting a potential role in neuroprotective function (Savaskan et al., 2007b). In mammals, the GPx4 protein maintains high stability and catalytic function in brain, even under conditions when selenium levels are compromised (Brigelius-Flohe, 1999). Knockout of *GPx4* in neural cells using *CamKIIα-Cre* transgenic mice resulted in increased lipid peroxidation leading to cell death (Seiler et al., 2008), whereas brain tissues from a mouse model of Alzheimer's disease showed reduced GPx4 associated with increased oxidized lipid by-products (Yoo et al., 2010). In addition to its role as an antioxidant enzyme, GPx4 modulates cell signaling and regulatory events (Imai and Nakagawa, 2003; Ufer and Wang, 2011; Ursini et al., 1997) including regulation of lipoxygenase and cyclooxygenase activity and apoptotic pathways (Chen et al., 2003; Huang et al., 1999; Imai et al., 1998; Schnurr et al., 1996; Seiler et al., 2008; Sutherland et al., 2001). Moreover, GPx4 has been shown to modulate the redox–regulated transcription factors nuclear factor-κB (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) (Brigelius-Flohe, 2006; Ufer and Wang, 2011). *In vitro* studies have suggested that GPx4 inhibits NF-κB activation and up-regulates heme oxygenase-1 via the Nrf2 pathway in vascular smooth muscle cells (Banning and Brigelius-Flohe, 2005). Interestingly, both NF-κB and Nrf2 are involved in heavy metal-induced stress responses (Chen et al., 2001; Liu et al., 2009; Lushchak, 2011). Collectively, the regulatory mechanisms of *gpx4* are consistent with a role in the maintenance of signal transduction processes as well as protecting against cellular oxidative stress during normal olfactory function.

Our hypothesis of a protective role of GPx4 in maintaining Coho olfactory function under oxidative stress was supported by the results of our chemical challenges in which we observed that exposure to Cd generally decreased *gpx4* mRNA expression in olfactory tissues. In this regard, Cd is a well-established olfactory toxicant in aquatic species (Blechinger et al., 2007; Matz and Krone, 2007) as well as in rodents (Bondier et al., 2008; Kumar et al., 1996; Sun et al., 1996). Exposure to Cd in mice leads to extensive lipid peroxidation in the mouse olfactory bulb which is accompanied by decreased levels of GSH (Kumar et al., 1996). Although the linkage between Cd-mediated oxidative damage and *gpx4* expression has not been well documented in fish, several studies have indicated a decrease in overall tissue GPx expression and/or catalytic activity following Cd exposure (Banni et al., 2011; Choi et al., 2007; Karaytug et al., 2011). Of relevance to the current project was a study showing decreased *gpx4a* mRNA expression in the olfactory lobes of common carp over a 96 hr period of Cd exposure (10 mg/L), accompanied by a complete elimination of *gpx4b* expression (Hermesz and Ferencz, 2009). The fact that we observed a loss of *gpx4* expression at low environmentally- relevant levels of Cd suggests that this phenomenon is of relevance to field scenarios and exposures encountered in polluted waterways.

Although there is little information concerning the translational control of *gpx4* in aquatic species, we have recently observed a general loss of several *GST* forms expression in Coho exposed to Cd (Espinoza et al., 2011). In our GST study, the loss of olfactory *GST* expression was accompanied by a strong and concomitant increase in the expression of metallonthionein, a sensitive marker of heavy metal exposures in fish (Bigot et al., 2011; Falfushynska et al., 2011; Gagne et al., 2007; Kim et al., 2010). In addition to observed effects at the mRNA level, Cd can inhibit GPx catalytic activities in several species, with the loss of catalytic capacity associated with GSH depletion or the formation of a chemical complex between Cd and selenium at the active site of the enzyme (Cuypers et al., 2010; Gambhir and Nath, 1992; Quig, 1998).

In addition to the comparably high levels of *gpx4* mRNA expression observed in liver and olfactory tissues, we also observed the presence of the *gpx4* paralogs in other tissues, including pyloric caeca, brain, heart, gonad, and kidney. While the functional significance of these differences are unknown, the broad tissue distribution of Coho *gpx4* is consistent with conservation of important physiological functions (Brigelius-Flohe, 1999; Schneider et al., 2006; Thompson et al., 2010; Zhang et al., 1989), such as protecting against free radical attack on lipid-rich membranes in these tissues. Others have reported that the comparably high *GPx4* expression in gonads is associated with a role in maintaining favorable redox status within the reproductive and endocrine systems, especially during sexual maturation or during spermatozoa development (Beckett and Arthur, 2005; Schneider et al., 2009).

In mammalian cells, the *GPx4* gene gives rise to three different isoforms that associate with different subcellular compartments, including mitochondrial GPx4 (m- GPx4), cytosolic GPx4 (c-GPx4), and nuclear GPx4 (n-GPx4). The alternative usage of the translational initiation site of *GPx4* genes controls the ultimate subcellular localizations of GPx4 proteins (Arai et al., 1999; Knopp et al., 1999; Maiorino et al., 2003; Nam et al., 1997; Pfeifer et al., 2001). The sequence between the first two initiation sites is thought to encode for a mitochondrial targeting sequence responsible for directing m-GPx4 import of the mitochondria (Thomas et al., 1990). Thus, m-GPx4 and c-GPx4 can not be distinguished at a protein level once this targeting sequence is cleaved. The fact that we observed total GPx activity and GPx4-specific catalytic activities in both the mitochondrial and cytosolic fractions of salmon tissues indicates subcellular targeting of GPx4 isoforms in salmon. However, in the present study we were unable to differentiate between the two GPx4 isoforms based upon substrate specificities. An existing structural model of the human GPx4 protein indicates that certain residues, including selenocysteine (U46), glutamine (Q81) and tryptophan (W136) are functionally critical to its catalytic activity (Scheerer et al., 2007). These three amino acids are considered a catalytic triad and are well conserved among fish and mammalian species, suggesting similar GPx4 catalytic activities or substrate specificities across species. Although GPx4-associated catalytic activities have not been extensively investigated in fish species, our studies in Coho are consistent with a similar catalytic profile with GPx4 in mammalian cells.

Summary and conclusions

In summary, we have cloned two paralogs of *gpx4* (*gpx4a* and *gpx4b*) from Coho salmon olfactory and liver tissues. The amino acid sequence of the two GPx4 isoforms shared 71% identity and exhibited high homology with other fish and mammalian GPx4 enzymes. Both isoforms showed a ubiquitous expression pattern among the tissues investigated, and catalytic function analysis substantiated relatively rapid initial rates of GPx4 enzymatic activity in olfactory and liver subcellular fractions. Accordingly, our results demonstrate a functional role of GPx4 in Coho salmon. Furthermore, the transient modulation of *gpx4* isoforms in liver and olfactory tissues on exposure to the prototypical environmental

contaminant Cd, while not showing an inductive or adaptive response, was suggestive of an overwhelming of cellular antioxidant responses by Cd. Our present study also extends observations in mammalian species indicating an important role of GPx4 in protecting against cellular oxidative damage in olfactory system of vertebrates. Of importance in future studies is the need to address the regulatory mechanisms of salmon *gpx4* in the context of chemical exposures, as well as functional studies addressing the role of *gpx4* knockdown on metal injury to the fish olfactory system.

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Highlights of the current research

- Cloned two *gpx4* isoforms (*gpx4a* and *gpx4b*) from the Coho salmon peripheral olfactory system
- Developed qPCR assays for a comprehensive analysis of *gpx4* expression in 10 tissues
- High initial rates of GPx4 enzymatic activity in Coho olfactory and liver tissues
- Examined the effect of cadmium on *gpx4* expression in olfactory and liver tissues

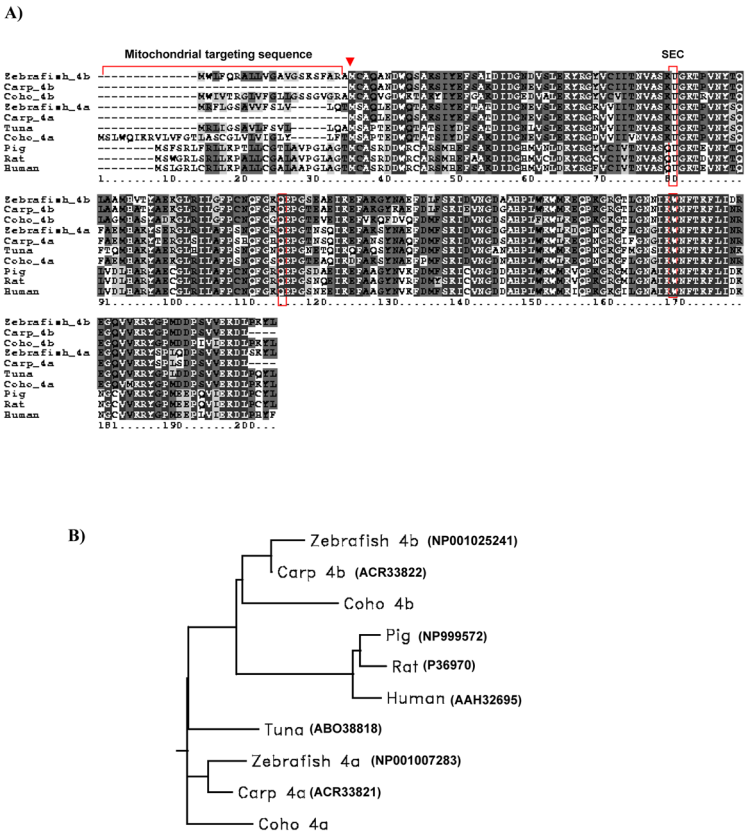

Figure 1.

(A) Predicted amino acid sequence alignment of the two Coho GPx4 isoform with other fish and mammalian GPx4s. The selenocysteine residue is indicated as U in sequence. The three boxes highlight conserved residues at selenocysteine (U), glutamine (Q) and tryptophan (W). Black shading indicates completely conserved residues and grey shading indicates similar residues (similarity threshhold fraction ≥0.7). The arrow indicates the second translational initiation site. Mitochondrial targeting sequences are underlined. (B) Phylogenetic rooted-tree showing the relationship of Coho GPx4 with other fish and mammalian GPx4s. GenBank accession numbers are shown in parentheses. Graph generated from San Diego Biology Workbench 3.2.

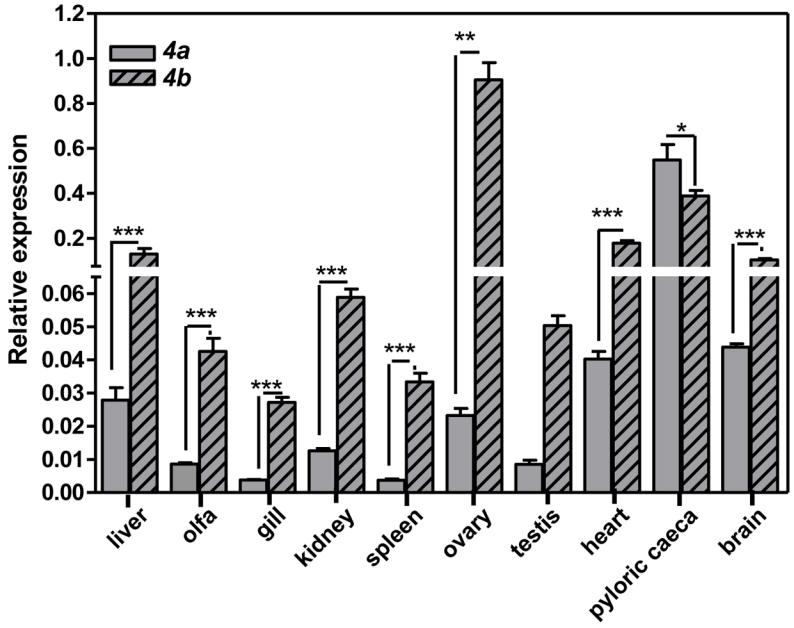

Figure 2.

Tissue-specific *gpx4* isoform expression. Gene expression normalized to β-actin. All data represent the mean ± SEM of measurement of 3 to 8 individuals. Asterisks indicate statistically significant difference compared to control samples (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$).

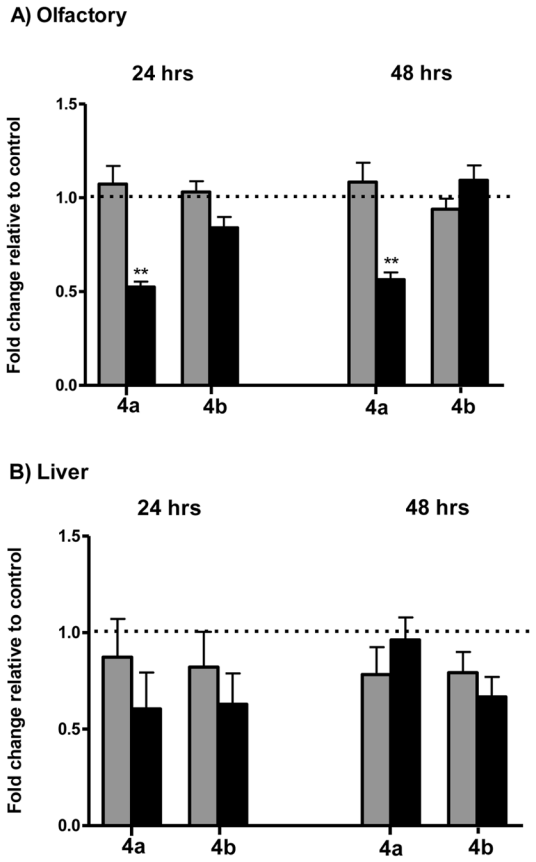

Figure 3.

Modulation of *gpx4* expression by Cd in Coho olfactory rosettes (A) and liver (B). Expression of *gpx4a* and *gpx4b* were measured in the olfactory rosettes and liver of Coho salmon exposed to 0, 3.7, and 347 ppb Cd for 24 and 48 hr periods. All data represent the mean ± SEM of n=8 individuals. Graphs are presented as fold-change relative to the corresponding controls, with the 3.7 ppb and 347 ppb datasets represented by light and dark bars, respectively. In the olfactory rosettes, data was normalized to a normalization factor comprised of multiple housekeeping genes as described in the methods. Liver mRNA data was normalized to the expression of β-actin. Asterisks indicate statistically significant difference compared to control samples ($^{**}p<0.01$).

Table 1

Primers for Coho *gpx4* cloning by RACE and for real-time PCR studies.

Oligo	Function	Primer (5′ to 3′)
1	*gpx4b* 5′Race reverse	CACCACCTGCCCTTCTCTGTTGATC
2	*gpx4b* 3′Race forward	GAATGCACGCCTCCTACGCTGACAA
3	*gpx4b* full-length	Forward: AGGATGTGGATCGTAACGCGTG
		Reverse: TCAGAGACATCACAGGTATTTAGG
4	*gpx4a* 3′Race forward	CACMGMYAGAGGACTGGC
5	*gpx4a* 5′Race reverse	ATCCTTCTCCACCACACTGG
6	*gpx4a* full-length	Forward: CAATTCAATGTCGTTGTGGC
		Reverse: GGATCTTCCTAAGTACCTGTAA
7	*gpx4b* RT primer	Forward: ATCACCAACGTTGCCTCTAAAT
		Reverse: CCTTGATTTCCACCTCTGTACC
8	*gpx4a* RT primer	Forward: GTACGCTGAGAAAGGTTTACGC
		Reverse: TTGATGCCATTTCCCAGG
9	β-actin RT primer	Forward: GACCCACACAGTGCCCATCT
		Reverse: GTGCCCATCTCCTGCTCAAA
10	GAPDH RT primer	Forward: TCTGTGTTGGAATCAACGGA
		Reverse: TGAAGAAGACTCCGGTGGAC
11	rpL9 RT primer	Forward: AAAAAGCTGCGTGTGGATAAAT
		Reverse: GATCGCATCTTATAGCGGAAAC

Table 2

Total GPx and GPx4-specific activity in Coho liver and olfactory rosettes using different hydroperoxide substrates*

Tissue	Substrate	Mitochondrial GPx or GPx4 (μmol/min per mg protein)	Cytosolic GPx or GPx4 (μmol/min per mg protein)
Liver[1]	CuOOH	28.4 ± 3.90	53.2 ± 3.51
	PCOOH	10.6 ± 0.53	12.3 ± 1.01
Olfactory rosettes[2]	CuOOH	62.6 ± 6.38	96 ± 7.72
	PCOOH	7.8 ± 1.70	9.1 ± 1.04

*Activities were measured as described under methods, section 2.4. The GSH concentrations in all assays were fixed as 3 mM.

[1]Enzymatic values in the liver tissue reflect the mean ± S.E. of 3 individual fish.

[2]Enzymatic values in the olfactory rosettes reflect the mean ± S.E. of n=3 pools.