Effects of Salinity on the Uptake, Biotransformation, and Toxicity of Dietary Seleno-L-Methionine to Rainbow Trout

Daniel Schlenk,*† Natalia Zubcov,† and Elena Zubcov†

*Environmental Toxicology Program, University of California, Riverside, California 92521, and †Institute of Zoology, Moldovan Academy of Sciences, Chisinau, Moldova

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Selenium (Se) is an element that has been of environmental concern in aquatic systems that drain arid regions heavily used for agricultural purposes. As hypersaline conditions are associated with these ecosystems, this study examined the effect of hypersaline water on the uptake, biotransformation, and toxicity of seleno-L-methionine (SeMe) in juvenile rainbow trout. Fish were acclimated for 5 days to four different salinity regimes (0.5, 6.3, 11.9, 16.8 decisiemens (dS/m) per meter. To mimic arid agricultural runoff solutes, the water was reconstituted with ions found in drainage water of the San Joaquin River in California. Following 7 days of dietary exposure to 180 mg/kg SeMe, mortality, as well as hepatic selenium concentrations and reduced:oxidized glutathione (GSH:GSSG) ratios were measured. Hypersaline conditions protected fish from dietary SeMe toxicity. Fish exposed to 0.5 dS/m water experienced 100% lethality in 2.5 days, whereas fish acclimated to 16.8 dS/m water-only experienced 16.7% mortality, which took 5–7 days to occur. There were no significant differences in hepatic selenium concentrations, but diminishment of reduced glutathione:oxidized glutathione (GSH:GSSG) ratios was observed in SeMe-treated fish held in 0.5 dS/m water. SeMe inhibited flavin-containing monooxygenase catalyzed trimethylamine oxidase activity, but salinity failed to induce expression in trout, indicating an oxygenation of organoselenides may play a minimal role in SeMe toxicity.

Key Words: selenium; selenomethionine; salinity; flavin-containing monooxygenase; glutathione; oxidative stress.

Selenium (Se) is an element that has been of environmental concern primarily in arid environments undergoing irrigation for agricultural purposes (Presser and Ohlendorf, 1988; Skorupa, 1998). Adverse effects on fish and birds have been noted as a result of dietary accumulation of organoselenides such as the amino acid conjugates selenomethionine, and selenocysteine, which are derived from transformations occurring within the food chain (Maier and Knight, 1994). The complex nature of the environmental fate and speciation of Se has challenged regulators to derive water quality thresholds for adverse biological effects in wildlife (Hamilton, 2002; Sappington, 2002). In addition, the mechanism(s) of toxicity of dietary Se has not been well documented. Recent studies indicate that understanding the speciation of the selenium within biological systems may help to better characterize the risk to fish and birds (Fan et al., 2002a). Several groups have indicated in birds and other mammals that organoselenides undergo extensive oxidation to selenoxides, which tend to consume cellular pools of the antioxidant glutathione (GSH), leading to oxidative stress and eventual toxicity (Chen and Ziegler, 1994; Spallholz, 1994). Threshold concentrations necessary to cause thiol depletion have yet to be calculated in any species.

Critical body burden thresholds for Se have been recommended as a method to assess ecological risk to susceptible wildlife (Hamilton, 2002). Unfortunately, one of the disadvantages of this approach is the poor understanding of the effects of other elements and contaminants on the fate and biological effects of selenium in wildlife. For example, deposition of solutes such as calcium, magnesium, and sulfate also occurs in waterways draining arid agricultural regions such as central California. Little is known about the combined effects of hypersaline water on the effects of Se in aquatic organisms.

Recent evidence in euryhaline organisms indicates that hypersaline conditions may exacerbate the toxicity of soft-nucleophilic chemicals, which are bioactivated by oxidation (El-Alfy et al., 2001; Wang et al., 2001). The thioether insecticide, aldicarb, is significantly more toxic to rainbow trout in saline water (El-Alfy and Schlenk, 1998; El-Alfy et al., 2001). The enhanced toxicity is apparently due to an induction of the flavin-containing monooxygenase (FMO) system by saline conditions (El-Alfy and Schlenk, 1998; El-Alfy et al., 2001). Induction of FMO enhanced the transformation of aldicarb to the more potent cholinesterase inhibitor, aldicarb sulfoxide, in rainbow trout and Japanese medaka, in which saline conditions increased toxicity (El-Alfy et al., 2001; Wang et al., 2001). Organoselenides have been shown to be excellent substrates for FMO, which produce the corresponding selenoxides (Chen and Ziegler, 1994; Ziegler, 1993). Thus, saline conditions could also potentiate the toxicity of organoselenides in these

1 To whom correspondence should be addressed. E-mail: daniel.schlenk@ucr.edu.

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309
species. The purpose of the current study was to test this hypothesis and to determine potential mechanism(s) by which selenium exerts its toxicity in fish.

MATERIALS AND METHODS

Chemicals. Unless otherwise noted, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO.).

Fish exposure. Juvenile rainbow trout (Oncorhyncus mykiss), 30-days post hatch, were obtained from the Mojave River Hatchery (California Department of Fish and Game, Victorville, CA) and maintained in chilled (18 ± 1°C) Riverside city water that was filtered to remove chlorine/chloramine. Hardness and alkalinity averaged approximately 200 mg/l CaCO₃ and the pH was 7.2. In 40-liter glass aquaria, two fish of approximately 30 g each were placed in 10 l of water. Animals were acclimated to saline water gradually by increasing the salinity approximately 0.3% per day to reach four salinity regimens (0.5, 6.3, 11.9, and 16.8 deciSiemens/meter [dS/m]) and then maintained for one week prior to dietary treatment to 180 mg/kg seleno-L-methionine (SeMe). This concentration of Se was chosen based upon preliminary range-finding studies demonstrating mortality within 96 h, and was approximately half of the maximum value found in Gambusia affinis from the San Luis drain in Kesterson Reservoir (Saiki and Lowe, 1987). The four salinity concentrations consisted of 3 dilutions of reconstituted water derived from the Westlands Water District located about 10 km south of Mendota in the San Joaquin River Drainage Basin (Saiki et al., 1992) (see Table 1 for ionic components), with Riverside city water as control. Salinity was determined by conductivity measurements and approximately corresponds to (0.23, 5.0, 9.5, and 13.4 g/l total suspended solids or parts per thousand salinity. Water was replaced daily and the conductivity (DS/m) measured prior to addition to aquaria. Water was added from stock dilutions and maintained at 18°C, using chilled water baths. The light/dark cycle was 16/8 h.

Animals were provided dietary SeMe for up to 7 days and were fed approximately 25 mg per fish. Fish weights averaged 5.1 ± 0.6 g and lengths were 6.5 ± 0.7 cm. Each salinity was tested in triplicate and the entire exposure was replicated a second time. Se-spiked food was prepared by thoroughly mixing dry commercial fish food (Nelson and Sons Silvercup; Murray, UT) with appropriate amounts of SeMe (Aldrich Chemical Company, Milwaukee, WI) in a methanol solution, to produce an Se concentration of 180 µg/g (dry weight), whereas the Se concentration in the food was 0.23 µg/g. The analysis of the fish diet showed that other elements in the food were present at typical concentrations (Table 2).

Tissue analysis of selenium. Samples of trout liver (0.5 g) were digested in nitric acid, hydrogen peroxide, and laboratory-distilled water at 170°C and ~200 pounds per square inch (psi) in a MARS 5 CEM microwave digester (Matthews, NC) for 30 min. In order to change the oxidative state of Se to Se(VI), the samples were heated at 90°C for 20 min in 12 ml HCl and 0.2 ml of 0.2 M K₂S₂O₇ to a final HCl concentration of 6 N. The samples were analyzed in a Perkin-Elmer atomic absorption spectrophotometer (Norwalk, CT).

For quality assurance and quality control, a dried tomato leaf reference material (National Institute of Standards and Technology; Gaithersburg, MD) and matrix spikes were analyzed with the samples. The percent recovery for the tomato leaves was 85%. The percent recovery for the spiked samples was >80%. The detection limit for the Se analysis ranged from 0.003 to 0.017 µg/g. All metal concentrations from fish were reported on a wet-weight basis, whereas food was measured as dry weight.

Hepatic GSH:GSSG. Total GSH and GSSG were measured using the method of Baker et al. (1990). Trout liver tissue samples were homogenized in 1/9 v/v 5% 5-sulfosalicylic acid (SSA) on ice. The homogenate was centrifuged and triplicate 10 ml samples of supernatant (~1.0 mg of tissue) or standard was added to a 96-well microplate. Two hundred µl of reaction buffer were then added to the wells for measurement of GSSG. Reaction buffer consisted of 5.40 ml NaH₂PO₄/EDTA buffer, 2.80 ml 1 mM DTNB (0.15 mM final), 3.75 ml of 1 mM NADPH (0.2 mM final) and 0.050 ml of 12U GSSG reductase (1 U per ml final). The microplate was read at 10-s intervals over two min at 405 nm at room temperature (25°C). The GSH assay was similar to the above, except that the reaction buffer did not contain NADPH or GSSG reductase.

Hepatic FMO expression and trimethylamine (TMA) oxidase activity. Microsomes from dissected livers of individual fish were prepared as previously described (Schlenk and Buhler, 1991). FMO protein expression was determined by immunoblot analysis using an anti-human FMO1 antibody, provided by Dr. A. Rettie (University of Washington). Western analysis showed that the antibody recognized only one band in liver. FMO activity was determined as trimethylamine oxidase using the method of Niizeki et al. (2002). Briefly, 1-ml incubations consisted of 1 mM NADPH, 10 mM Potassium Phosphate (pH 8.0), and 0.5 mg of microsomal protein. Incubations were carried out at 25°C for 30 min following the addition of substrates 100 µM C₄-trimethylamine (1 mMCl/mmol) and/or 100 µM selenomethionine in methanol. Negative control incubations were initiated with methanol only, or lacking NADPH.

Data analysis. Trout liver FMO expression, Se concentrations, and GSH: GSSG ratios, were analyzed with a two-way analysis of variance (ANOVA) to...
determine the statistical differences between the salinity and SeMe treatments (\(p < 0.05\)). Dunnett’s test was performed if significance among different means was observed, to determine which treatment groups were significantly different from the control. TMA oxidase and FMO expression activities were compared using Student’s \(t\)-test.

RESULTS

Fish maintained in the higher-saline water were resistant to 180 mg/kg dietary SeMe treatment and experienced limited (33 and 0%) mortality compared to animals treated in unamended freshwater (0%) (Table 3). Time to death was significantly different (\(p \leq 0.05\)) in the fish exposed to the salinity regimens with a dose-dependent effect observed (Table 4). All animals maintained in 0.5 dS/m water died within 1–3 days (2.5 average), whereas animals in 16.8 dS/m water were still alive for up to 7 days of continuous dietary treatment with SeMe.

Hepatic concentrations of Se were measured in all of the treated animals and were not significantly different (Table 4). In contrast, Hepatic GSH:GSSG ratios were significantly reduced only in animals fed SeMe and maintained in the unamended water (Table 5). There were no significant differences in reduced GSH or total GSH concentrations of the livers from fish in any other treatment. However, GSSG levels were significantly higher in the SeMe-treated animals of the unamended water.

FMO activity was measured in untreated fish. TMA oxidase in liver microsomes was significantly inhibited by coincubation of equimolar SeMe. Values of microsomal TMA oxidase from fish held in unamended water were 0.77 ± 0.24 nmol/min/mg, whereas when microsomes were coincubated with SeMe, TMA oxidase values diminished to 0.46 ± 0.020 nmol/min/mg. Due to limited tissue, expression of hepatic FMO was measured in trout in control and the highest-salinity regimen. Expression was unaltered by the saline water (data not shown).

DISCUSSION

Postulated mechanisms of organoselenenide toxicity include disruption of tertiary protein structure via sulfi de replacement and disruption of tertiary protein structure via sulfi de replacement (Fan et al., 2002b) and oxidative stress through either futile redox cycling (Spallholz, 1994). Although several selenium species are capable of initiating the oxidative process without biotransformation, oxygenation of organoselenenides to selenoxides have been shown to consume glutathione as well as other thiols (Chen and Ziegler, 1994). The enzyme(s) responsible for the initial oxidation has yet to be determined, but in vitro studies with FMOs in mammals have indicated that organoselenenides are excellent substrates with selenomethionine as well as dimethylselenide and selenocysteine all undergoing oxidation with relatively high turnover rates (Chen and Ziegler, 1994; Rooseboom et al., 2001). In trout liver microsomes, SeMe significantly inhibited the oxidation of TMA, which has been shown to be a substrate for FMO in trout as well as other fish species (Baker et al., 1963; Schlenk, 1993, 1998). As inhibition of FMO can only occur competitively (Ziegler,
SeMe is likely a substrate for the trout liver enzyme system. Studies are currently underway to confirm these indirect measurements by the identification and quantification of the putative selenoxides metabolite formed by FMO.

FMOs are uniquely regulated in rainbow trout and are induced by hypersaline conditions (Larsen and Schlenk, 2001). However, all studies demonstrating induction were performed using waters dominated by Na and Cl, which are typical in seawater systems. In drainages of arid regions where Se contamination has been observed, waters are also saline. In contrast to marine or seawater ions, arid drainages tend to be dominated by Ca, Mg, and sulfate ions (Saiki et al., 1992). It was our hypothesis that saline water could induce FMO(s) leading to elevated activation of organoselenides to the corresponding selenoxide initiating redox cycling, oxidative stress, and eventual toxicity.

Although SeMe did appear to be a substrate for trout liver FMO, saline conditions provided protection against toxicity following dietary exposure to SeMe rather than enhancing toxicity. Previous studies have shown that sulfate-dominated water significantly protected biota from acute toxicity following aqueous exposure to inorganic selenate (Brix et al., 2001; Forsythe and Klaine, 1994; Maier et al., 1993; Ogle and Knight, 1996). Reduction of aqueous Se uptake into the cell was observed through the ability of sulfate to limit the bioavailability of Se at one or two transporters for which sulfate and selenate compete (Hansen et al., 1993). However, this mechanism may not be relevant in upper level predators such as predatory fish, where Se exposure occurs via dietary exposure.

The failure of the saline water to alter hepatic selenium concentrations were consistent with those of other groups who demonstrated that sulfate did not affect selenite or SeMe accumulation or toxicity in aqueous laboratory or longer-term microcosm experiments (Besser et al., 1989; Maier et al., 1993). Concentrations observed in the liver exceeded proposed body burden thresholds for teratogenic or reproductive endpoints (4 mg/kg) (Hamilton, 2002). Consequently, since saline water protected fish from toxicity but did not alter hepatic levels of selenium, other mechanisms besides reduction of selenium uptake were responsible for the observed resistance.

Oxidative stress, in the form of diminished hepatic GSH: GSSG ratios, was observed only in SeMe trout that died more rapidly than trout held in higher-saline water. The reduction of the ratio was due to enhanced GSSG rather than depleted GSH. As discussed earlier, GSH depletion and subsequent oxidative damage have been observed in avian species by various inorganic and organoselenium species (Spallholz, 1994).

GSH was oxidized in fish maintained in freshwater, but values were unchanged in fish maintained in all saline conditions. Since sulfate is a necessary precursor for GSH synthesis, one potential mechanism of protection by saline water may have been augmentation of cellular thiols such as GSH in the liver or some other extrahepatic organ with subsequent transport to the liver. However, studies in mammals have demonstrated that treatment with sulfate did not significantly alter cellular GSH concentrations nor provide protection against toxicants that induce oxidative stress in the liver (Hjelle et al., 1986). Evidence in channel catfish also argues against a labile pool of GSH available for movement between tissues (Gallagher et al., 1992).

Other possibilities include augmentation of hepatic or extrahepatic GSH by osmotic pressure (Larsen and Schlenk, 2002), reductions in bile flow, or diminished GSH reductase activity. Although several other studies have observed exclusive increases in GSSG without concomitant changes in GSH in other animals exposed to various selenium species, the majority of affected animals showed concurrent reductions in reduced GSH and increases in GSSG (see Hoffman, 2002, for review). It is also interesting to note that whereas several selenium species such as selenocysteine and selenite produce GSH-consuming superoxide anion radical in vitro, SeMe was unable to produce the radical in the same cell-free system (Spallholz 1994, 2001). However, these experiments did not have an oxidizing enzyme (i.e., FMO) present, which would have formed selenoxides that deplete GSH (Chen and Ziegler, 1994).

In addition to oxidative damage, Se toxicity, particularly in reproductive targets, has been observed to correlate to its binding to proteins in these targets (Fan et al., 2002b). Fan et al. (2002a) have identified at least five selenium-containing proteins in largemouth bass eggs that are upregulated in fish residing within selenium contaminated waterways and may be associated with toxicity. Thus, one potential explanation for the protective effects of saline conditions may involve the ability of sulfate, calcium, or magnesium to prevent the interaction of SeMe with critical subcellular proteins by maintaining the cellular redox potential. This would be consistent with the lack of FMO upregulation by sulfate-dominating water, since FMO is likely involved in maintaining cellular redox potential (Larsen and Schlenk, 2002; Suh and Robertus, 2000). Further study into the fate and subcellular distribution of sulfate, calcium, and magnesium with molecular targets of Se toxicity, as well as cellular redox maintenance pathways, are necessary to better understand the mechanisms of selenium toxicity as well as the protection afforded by saline conditions in arid drainages.

In summary, contrary to previous studies showing bioactivation of pesticides by saline conditions, saline water derived from arid drainages of central California provided significant protection to juvenile trout fed SeMe. Survival was not associated with hepatic selenium concentrations. Although SeMe is a substrate for hepatic FMO in the trout, FMO expression was unaltered by salinity treatment indicating a mechanism(s) of protection that does not involve selenoxide formation, but may involve sulfate, Ca, or Mg mediating other cellular processes involved in enhancing redox status or altering subcellular distribution of selenium.
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REFERENCES


