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Lipid peroxidation and oxidative stress responses of salmon fed a diet containing perfluorooctane sulfonic- or perfluorooctane carboxylic acids

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ABSTRACT

The present study was conducted to evaluate the effects of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) on lipid β-oxidation and oxidative stress responses in Atlantic salmon liver and kidney tissues. We quantified changes in the expression levels of peroxisome proliferator-activated receptors (PPARs) and acyl-CoA oxidase (ACOX1) enzyme whose transcription is induced by PPARs. In addition, we analyzed gene expression patterns for enzymatic antioxidants (superoxide dismutase: SOD, catalase: CAT and glutathione peroxidase: GPx). Thiobarbituric acid reactive substances (TBARS) were analyzed as a measure for lipid peroxidation. Juvenile Atlantic salmon were repeatedly force-fed food spiked with PFOA or PFOS at 0.2 mg/kg, and samples were collected after 0, 2, 5 and 8 days and after a 7 days recovery period. Our data showed that exposure of salmon to PFOS or PFOA produced changes (either increased or decreased) in mRNA expression for PPARs, ACOX1, oxidative stress responses and lipid peroxidation (TBARS) and these responses showed marked organ differences, associated with tissue bioaccumulation patterns and dependent on exposure time. Given that a classical reaction during reactive oxygen species (ROS)-induced damage involves the peroxidation of lipids, our study demonstrates that salmon continuously exposed to dietary PFOS or PFOA dose showed alteration in peroxisomal responses and oxidative stress responses, with higher severity in the kidney, compared to liver. Overall, our data suggest that ROS-mediated oxidative damage maybe a significant and putative toxic effect of PFOA and PFOS in fish as has been reported in mammals.

1. Introduction

Perflourinated compounds (PFCs) are global environmental contaminant and are highly bioaccumulative in exposed biota including humans, making them contaminants of particular environmental concern (Inoue et al., 2004; Harada et al., 2007; Gebbink et al., 2009). 7PFCs have a widespread application in a myriad of products, including household cleaning agents, non-stick cookware, carpets, textiles, paper coatings, cosmetics, fire-fighting foams and packaged-food containers (Kissa, 1994; Hekster et al., 2003; Lehmler, 2005; Betts, 2007). Although PFCs and their precursors with C_8 chain lengths were apparently phased out in 2002, perfluorooctane sulfonate (PFOS), and to a lesser extent perfluorooctanoic acid (PFOA), continue to be particularly persistent and bioaccumulative in wildlife and fish (Houde et al., 2006). The general concentrations of PFOS and PFOA in the aquatic environment are usually low (1–100 ng/L), but the compounds have the potential to bioaccumulate through the food chain (Giesy and Kannan, 2002; Hansen et al., 2002; Nakayama et al., 2005). PFOA has been detected in surface and ground waters in North America, Europe and South-East Asia (Hansen et al., 2002; Martin et al., 2004; So et al., 2004; Van de Vijver et al., 2004). In fish, PFCs enter the organism through the gills or ingested food, and are transported after binding to plasma proteins such as albumin (Zhang et al., 2009).

Several vertebrates and non-vertebrates studies have indicated that PFOA and PFOS have the potential to act as peroxisome proliferators (PPs) (Bjork and Wallace, 2009). Peroxisomes are organelles that produce and degrade hydrogen peroxide (H_2O_2) and are involved in fatty acids (FAs) metabolism (Green, 1995) and these biological functions are mediated through the peroxisome proliferator-activated receptors (PPARs). Peroxisomes also contain oxidative enzymes, such as CAT, D-amino acid oxidase and uric acid oxidase (Motojima, 2000). PPs may also increase the transcription of genes in the peroxisomal β-oxidation enzyme system and inhibit secretion of very low-density lipoproteins (VLDL) and cholesterol from the liver (Solaas et al., 2004). Thus, PPARs regulate cellular differentiation and energy homeostasis in higher organisms, where they are known to exhibit broad, isotype-specific tissue expression patterns. For example, while PPARα is expressed at high levels in organs with significant catabolism of fatty acids, PPARβ and PPARγ have the broadest expression pattern, and their levels of expression in certain tissues depend on the extent of cell proliferation and differentiation (Michalik et al., 2006; Rakhshandehroo et al., 2007). In mouse, it

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has been shown that PFOA-induced developmental toxicity requires the activation of the PPARα. Furthermore, there are conflicting data regarding the ability of PFOA and other PFCs to induce expression of PPARα controlled genes in aquatic organisms (Ren et al., 2009).

Increased hydrogen peroxide (H_2O_2) production due to exaggerated fatty acid β-oxidation may be harmful for the organism (Bonekamp et al., 2009). These may cause deleterious cellular effects, such as cell death and other pathological conditions as a result of reactive oxygen species (ROS) produced during chemical or oxidative stress conditions (Bonekamp et al., 2009). The enzymes, SOD, CAT and GPx play significant roles as antioxidants and their elevated expression and activity are indicative of oxidative stress (Lushchak et al., 2001). An optimal adaptation during limited oxygen requires the induction of antioxidant and associated enzymes, such as CAT, SOD and GPx, in order to reduce potential damage during oxygen reintroduction, such as during lipid peroxidation (Storey, 1996). Excessive rate of ROS formation as a result of exposure to environmental pollutants may exceed the antioxidant capacity and subsequently result to oxidative stress in organisms (Hermes-Lima and Storey, 1998). Thus, organisms are able to adapt to ROS formation by increasing the expression of antioxidant enzymes, in addition to many other forms of defence and repair of oxidative damages (Gonzalez-Flecha and Demple, 2000).

The aim of the present study was to evaluate the effects of PFOA and PFOS on lipid β-oxidation system of salmon liver and kidney tissues by investigating the transcriptional and translational expression of PPARs, which has been shown to be peroxisome proliferator inducible, and mRNA of the acyl-CoA oxidase (ACOX1) enzyme, whose transcription is induced by PPARs. We quantified thiobarbituric acid reactive substances (TBARS) as a measure of peroxisomal lipid β-oxidation. In addition, we analyzed gene expression patterns for enzymatic antioxidants (SOD, CAT and GPx). Our hypothesis is that exposure of juvenile Atlantic salmon (*Salmo salar*) to PFOS or PFOA through food will produce differences in the organ expression patterns for genes and enzymes involved in lipid peroxidation and oxidative stress and will be indicative of potential adverse physiological and health effects.

2. Materials and methods

2.1. Chemicals

PFOA and PFOS (linear, technical grade) were purchased from Alfa Aesar (Karlsruhe, Germany). Trizol reagent for ribonucleic acid (RNA) purification and TA Cloning kit were purchased from Invitrogen Corporation (Carlsbad, CA, USA). iScript cDNA synthesis kit, iTAQ™-SYBR® Green Supermix with ROX, an Immun-Star WesternC Chemiluminescent Kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Fish, treatments and sampling

Juvenile Atlantic salmon (*Salmo salar*) with average mass of 70 ± 20 g were obtained from Åsen settefisk (Nord-Trøndelag, Norway). The fish were sexually immature and contained a random sample of male and female fish. They were maintained in 300 L tanks at 10 °C with running water under a photoperiod of 14:10, light:dark at the Department of Biology, Norwegian University of Science and Technology (NTNU) animal holding facilities. In order to ensure equal exposures of PFOA and PFOS between individuals, the fish were randomly distributed to exposure tanks and fed gelatine capsules containing fish-feed spiked with PFOA or PFOS (each at 0.2 mg/kg fish) or solvent (methanol: 0.01% by volume). In addition, another group receiving gelatine capsules without solvent was included in the experimental setup. The gelatin capsules were kept at room temperature for 6 h to evaporate the solvent (methanol) prior to feeding. The PFOA and PFOS doses was chosen based on available information in the literature and observed levels in the environment and biota (Hansen et al., 2001; Giesy and Kannan, 2002). The capsules were force-fed (oral gavage) at days 0, 3 and 6 followed by 7 days of depuration period. Six individuals ($n = 6$) were collected at days 2, 5, 8 and 14 (end of depuration period). It should be noted that samples were collected 2 days post-feeding with the capsules. Liver and kidney were divided and processed for gene expression, protein and TBARS analyses (see below). One exposure dose was used because oral gavage is a time consuming and tedious exposure method. This route of exposure was chosen against waterborne exposure to guarantee full bioavailability of the compound as it was expected that substantial amount of these compounds would adhere to tank surface due the unique chemistry of PFCs and significantly reduce the amount of bio-available concentration.

2.3. Gene expression analysis

Total cDNA for the gene expression analyses were generated from 1 μg total RNA from all samples using a combination of poly-T and random primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad). High quality RNA with A260/A280 ratio above 1.9 and intact ribosomal 28S and 18S RNA bands was used for cDNA synthesis. Quantitative (real-time) PCR was used for evaluating gene expression profiles. For each treatment, the expression of individual gene targets was analyzed using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA). Each 25 μL DNA amplification reaction contained 12.5 μL of iTAQ™SYBR Green Supermix with ROX (Bio-Rad), 1 μL of cDNA, and 200 nmol of each forward and reverse primer (Table 1). The three-step real-time PCR program included an enzyme activation step at 95 °C (3 min) and 40 cycles of 95 °C (15 s), 60 °C (30 s), and 72 °C (20 s). Controls lacking a cDNA template were included to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. The criterion for using the standard curve is based on equal amplification efficiency (usually >90%) with unknown samples and this is checked prior to extrapolating unknown samples to the standard curve (Arukwe, 2006). The standard plots were generated for each target sequence using known amounts of linear plasmids containing the amplicon of interest. Data obtained from six fish were averaged and expressed as percentage of control samples.

2.4. Measurement of TBARS

As an index of lipid peroxidation, malondialdehyde (MDA) levels in kidney and liver were measured. MDA reacts with thiobarbituric acid as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex with peak absorbance at 532 nm as described previously (Dawn-Linsley et al., 2005). Briefly, liver and kidney tissue was homogenized (20%, w/v) in a 50 mM Tris–HCl buffer (pH 7.6) containing 150 mM NaCl, 1% Tergitol (NP-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS) and centrifuged at 3000*g* for 10 min, then mixed with ice cold 10% Trichloroacetic acid (TCA) to precipitate proteins. After centrifugation at 2000*g* for 15 min, 200 μL of sample was mixed thoroughly with equal volumes of 0.67% (w/v) of thiobarbituric acid and heated at 95 °C for 10 min in a water bath. The development color intensity was measured at 532 nm using a spectrophotometer. The MDA standard curve was constructed over the concentration range of 0–10 mM. Total protein concentrations in samples were determined with the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard. TBARS results were expressed as nmol MDA/mg protein.

2.5. Statistical analysis

Statistics was performed with GraphPad Prism, version 5 (GraphPad Software Inc). Significant differences between control and exposure