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Dietary lipids modulate methylmercury toxicity in Atlantic salmon

Pål A. Olsvik *, Heidi Amlund, Bente E. Torstensen

National Institute of Nutrition and Seafood Research, Nordnesboder 1-2, N-5005 Bergen, Norway

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ABSTRACT

This experiment aimed to study the molecular toxicity of methylmercury (MeHg) in liver, brain and white muscle of Atlantic salmon fed a diet based on fish oil (FO, high dietary $n-3/n-6$ ratio) compared to an alternative diet mainly based on vegetable oil (VO, low dietary $n-3/n-6$ ratio). Juvenile salmon were fed decontaminated diets or the FO and VO diets enriched with 5 mg Hg/kg (added as MeHg) for three months. The dietary lipid composition affected the fatty acid composition in the tissues, especially in liver and white muscle. After 84 days of exposure, the liver accumulated three times as much MeHg as the brain and white muscle. Vitamin C content and heme oxygenase, tubulin alpha (TUBA) and Cpt1 transcriptional levels all showed significant effects of MeHg exposure in the liver. TBARS, α-tocopherol, γ-tocopherol, and the transcriptional levels of thioredoxin, heme oxygenase, TUBA, PPARB1, D5D and D6D showed an effect of dietary lipid composition in liver tissue. Effects of dietary lipids were observed in brain tissue for MT-A, HIF1, Bcl-X and TUBA. Interaction effects between MeHg exposure and dietary lipid composition were observed in all tissues. Our data suggest that dietary fats have modulating effects on MeHg toxicity in Atlantic salmon.

1. Introduction

Methylmercury (MeHg), the most toxic form of mercury (Hg), is a common environmental contaminant formed when inorganic Hg is methylated by bacteria. In the marine environment, MeHg accumulates in organisms and biomagnify in the aquatic food chains. As a result, the highest concentrations of MeHg are often found in piscivorous fish and top marine predators. MeHg also represents an environmental problem in freshwater habitats, with thousands of lakes and rivers worldwide being seriously polluted with methylmercury (Grandjean et al., 2010), often followed by local food consumption advices (USEPA, 2009). Nearly all (95–99%) Hg in contaminated fish is MeHg (Grieb et al., 1990; Bloom, 1992). Fish consumption represents the main source of MeHg for humans, with many studies suggesting a direct correlation between body burden of MeHg and fish consumptions (Morel et al., 1998; Chapman and Chan, 2000; Cole et al., 2004; Bjornberg et al., 2005; Diez, 2009). In humans, MeHg is a well-known neurotoxicant, particularly affecting the developing nervous system, and has been associated with many different neurological problems (Davidson et al., 2010). The cytotoxicity of MeHg has been attributed to three major mechanisms: (A) disturbance of intracellular Ca^{2+} levels, (B) induction of oxidative stress by either production of excessive free reactive oxygen species (ROS) or by depleted oxidative defense capacity and (C) interactions with sulfhydryl groups by disrupting function of proteins and peptides containing cysteine and methionine (Ceccatelli et al., 2010). Neurons are thought to have low glutathione activity, making them especial susceptible to mercury insult.

Fish studies suggest that MeHg may affect reproductive hormones, mediating smaller gonad development or atrophy, delays in spawning and effects on fecundity (Klaper et al., 2006, 2008). Apoptosis was also suggested to be the main effect of MeHg exposure in liver, brain and muscle tissues of zebrafish (*Danio rerio*) (Gonzalez et al., 2005). Apoptotic cell death induced by MeHg has also been reported in mammalian studies, with signaling pathways such as caspase-, bax-, calpain-, cytochrome c release and lipid peroxidation activation being involved (Ceccatelli et al., 2010). Caspase genes were up-regulated in liver in response to both acute and chronic MeHg exposure in fathead minnow (*Pimephales promelas*), whereas the transcription of fatty acid synthase was down-regulated (Klaper et al., 2008). In human colonic cancer cells, inhibition of fatty acid synthase has been shown to induce apoptosis (Huang et al., 2000). Klaper et al. (2008) further showed that MeHg exposure affected the transcriptional levels of immune system genes both in gonads and liver of fathead minnows, with antigen-presenting genes, i.e. MHC class I genes, showing the strongest response. In brain of zebrafish acutely exposed to MeHg, microarray analysis suggested involvement of oxidative stress and effects on protein structure in the mechanism of action of MeHg (Richter et al., 2011). In a genome-wide study using zebrafish, Cambier et al. (2010) showed that dietary MeHg exposure for 25 days affected the transcription of genes encoding proteins

* Corresponding author. Tel.: +47 41459367; fax: +47 55905299.
E-mail address: pal.olsvik@nifes.no (P.A. Olsvik).

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involved in protein synthesis (ribosomal protein genes), and oxidative and endoplasmic reticulum stress in skeletal muscle. Their study also suggested that MeHg exposure affect lipid metabolism, calcium homeostasis, iron metabolism, muscle contraction and cell cycle regulation in muscle tissue. Berg et al. (2010), using a proteomic approach, showed that MeHg exposure for two-weeks affected mechanisms such as mitochondrial dysfunction, oxidative stress, altered calcium homeostasis, and disruption of microtubules in brain of Atlantic cod (*Gadus morhua*). Thus, oxidative stress and apoptosis seem to be two of the major mechanisms affected by MeHg treatment in fish.

It is well known that dietary factors such as selenium, zinc, cysteine, proteins, fats, fibers and vitamins interact with the metabolism of Hg at the physiological level and thereby modulate Hg toxicity. Nutrients may affect bioavailability, toxicodynamics and transport to target organs, and influence functional responses to Hg (Chapman and Chan, 2000). Jin et al. (2007, 2008) showed that a fish oil diet rendered greater sensitivity to effects of MeHg on the immune system and oxidative stress in rats while at the same time offering increased protection on other systems, suggesting significant modulating effects of dietary fats on MeHg toxicity in animals. Even so, the underlying mechanisms behind the interactions between diet and MeHg remain surprisingly unknown.

Farmed fish like Atlantic salmon (*Salmo salar*) may contain Hg due to the contents of MeHg in dietary fishmeal. In fish like Atlantic salmon and Atlantic cod dietary MeHg is mainly accumulated in fish muscle, where it is incorporated into larger peptides or proteins (Amlund et al., 2007). During the last decade vegetable oils have become a common ingredient in Atlantic salmon feeds (Turchini et al., 2009). While fish oils have relatively large amounts of long chained unsaturated fatty acids, including eicosapentaenoic acid (EPA, 20:5$n-3$) and docosahexaenoic acid (DHA, 22:6$n-3$), vegetable oils are generally dominated by 18 carbon fatty acids; oleic acid (18:1$n-9$), linoleic acid (18:2$n-6$) and linolenic acid (18:3$n-3$). Dietary fatty acid composition is known to affect tissue fatty acids in Atlantic salmon however to a varying degree dependent on type of tissue (Torstensen et al., 2004). Tissues like brain and retina are less influenced by dietary fatty acid composition compared to e.g. muscle and liver (Brodtkorb et al., 1997; Torstensen et al., 2004). The impact of altered tissue fatty acid composition in fish on MeHg toxicity is largely unknown and more research is needed into possible interactions between nutrients and contaminants in fishes.

The aim of this experiment was therefore to study the molecular toxicity of MeHg in liver, brain and white muscle of Atlantic salmon fed a diet based on fish oil (FO, high dietary $n-3/n-6$ ratio) compared to an alternative diet based on vegetable oil (VO, soybean oil, low dietary $n-3/n-6$ ratio). First, we wanted to determine whether the lipid composition and accumulation of MeHg in liver, brain and muscle reflect the dietary levels and to study possible interactions between dietary lipids and MeHg exposure, and secondly whether these possible differences affected the transcriptional levels of a selected set of MeHg exposure marker genes. We hypothesized that the altered tissue fatty acid composition imposed by these diets may affect MeHg toxicity at the transcriptional level in the fish.

2. Materials and methods

2.1. Materials and experimental set up

The feeding trial was carried out at the Matre Aquaculture Research Station (Matredal, Norway; 60°52′N, 05°35′E) during the period 23rd of April 2008 to 15th of July 2008. Atlantic salmon ($n = 825$) with an initial weight of 340 ± 17 g (mean ± SD, $n = 27$) were randomly distributed to 15 fiberglass tanks (1.5 × 1.5 × 0.9 m, water depth 0.6 m) with 55 individuals in each tank. The tanks were provided with a continuous flow of seawater with an average salinity of 35‰ and an average temperature 8 °C during the experimental period. The light regime was 12 h light and 12 h dark. Triplicate tanks of fish were fed one of four experimental diets, produced by EWOS Innovation (Dirdal, Norway). All diets contained the same 6 mm base pellet and either cleaned fish oil (FF Skagen, Skagen, Denmark) or refined soybean oil (Mills, Oslo, Norway) as lipid source. The four experimental diets were fish oil (FO) or soybean oil (VO) with or without addition of MeHg (methylmercury(II) chloride, CH_3ClHg; Riedel-de Haën, Sigma–Aldrich, Seelze, Germany), 5 mg Hg/kg to the vegetable oil diet (5VO) and 5 mg Hg/kg (5FO) to the fish oil diet. All lipids were added to the base pellet during coating. Methylmercury chloride was dissolved in a small volume of 100% ethanol (Absolutt alcohol prima; Arcus kjemi AS, Vestby, Norway) and mixed with the oils before coating. The final Hg concentrations of the diets were 0.08 ± 0.0 (mean ± SD, SD < 0.05) mg Hg/kg for VO and FO, 5.7 ± 0.6 mg Hg/kg for 5VO and 5.2 ± 0.6 mg Hg/kg for 5FO ($n = 3$ for all diets). In the VO and FO feeds, MeHg constituted 62–90% of the total Hg, whereas MeHg constituted >80% of the total Hg in the other diets. There were no differences in proximate composition of the four diets (Supplementary data Table 1).

2.2. Fish sampling

Fish were sampled the first day (initial sampling, 9 individuals), after seven days (36 individuals) and after three months (84 days, 36 individuals) of feeding the experimental diets. The fish were not fed 24 h prior to the samplings. From each tank, six fish were randomly sampled and immediately killed by a blow to the head, without damaging the brain. Weight (Table 1) and length of the fish were recorded and blood samples were taken before the tissues (liver, brain and white muscle) were dissected out. Samples were immediately flash frozen in liquid nitrogen and stored at −80 °C until further analysis. Samples were pooled from 6 individuals from each tank for lipid, protein and vitamin analyses, and with triplicate tanks, these measurements ($n = 3$) represents quantitative data from 18 individuals. Hg and transcriptional analyses were done on individual fish tissue samples, except for Hg in liver, which were analyzed in pooled samples. Blood samples for plasma cortisol measurements were extracted from the caudal blood sinus with a heparinized syringe. Plasma was separated from the blood by centrifugation (2000*g*, 10 min, 4 °C).

2.3. Mercury determination by inductively coupled plasma mass spectrometry (ICPMS)

Total Hg content was analyzed by ICPMS following microwave-assisted acid decomposition as described by Amlund et al. (2007). In short, approximately 0.4 g wet sample were weighed accurately into a 100 ml Teflon© digestion vessels (Milestone Inc., Shelton, CT, USA). Two ml HNO_3 (65%, Suprapur; Merck, Darmstadt, Germany) and 0.5 ml H_2O_2 (30%; Merck, Darmstadt, Germany) were added to the vessels, and the mixtures were digested in a microwave digestion system (Ethos1600; Milestone, Sorisole, Italy) using the following program: 250 W for 1 min, 0 W for 1 min, 250 W for 5 min, 400 W for 5 min and 650 W for 5 min. Samples were then diluted with deionized water (>17 M/cm; Nanopure System; Barnstead, Dubuque, IA, USA), brain samples were diluted to 10 ml, muscle samples and reference materials to 25 ml.

Total Hg concentrations in all samples were determined by ICPMS (Agilent ICPMS 7500c; Yokogawa analytical systems, Tokyo, Japan) equipped with an autosampler ASX-500 (CETAC Technologies, Omaha, NE, USA). Data were collected and processed using the Agilent Chemstation ICPMS software (Agilent Technologies, Palo Alto, CA, USA). Rhodium was used as an internal standard to correct for drift of the instrument. The accuracy of the method was assessed by analysis of two certified reference materials; oyster tissue (SRM 1566b; National Institute of Standards and Technology, Gaithersburg, MD, USA; certified value 0.0371 ± 0.0013 mg/kg; obtained value 0.037 ± 0.004, $n = 12$), and lobster hepatopancreas (TORT-2; National Research Council, Ottawa, Canada, certified value 0.27 ± 0.06; obtained value 0.32 ± 0.01, $n = 11$).

Mercury speciation analysis was performed on diets and muscle tissue as previously described (Amlund et al., 2007). In short, homogenized samples were dissolved in tetramethylammonium hydroxide (Merck, Darmstadt, Germany), extracted in toluene (Merck) and derivatized with a Grignard reagent (butylmagnesium chloride in tetrahydrofuran; Aldrich, Steinheim, Germany). Mercury species were separated by gas chromatography (Agilent 6890 NCG; Agilent Technologies, equipped with an injector Agilent 7683 and an autosampler Agilent G2614A; Agilent Technologies, Palo Alto, CA, USA) and detected using ICPMS (Agilent ICPMS 7500a, Yokogawa analytical systems, Tokyo, Japan). Data were collected and processed using the Agilent Chemstation ICPMS chromatographic software (Agilent Technologies, Palo Alto, CA, USA). Accuracy of the method was assessed by analysis of two certified reference materials; oyster tissue (SRM 1566b; National Institute of Standards and Technology, Gaithersburg, MD, USA; certified value 0.0132 ± 0.0007 mg/kg; obtained value 0.017 ± 0.003, $n = 4$) and lobster hepatopancreas (TORT-2; National Research Council, Ottawa, Canada, certified value 0.152 ± 0.013; obtained value 0.161 ± 0.003, $n = 4$). The contribution of MeHg to all Hg was estimated by comparing the concentration of MeHg to the total Hg concentration.