Interactions between marine n-3 fatty acids and methylmercury

in Atlantic salmon (Salmo salar)

Ole Jakob Nøstbakken

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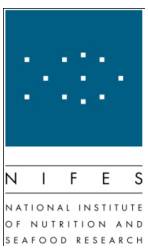

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Abstract

Methylmercury (MeHg) is an environmental contaminant widely distributed in the aquatic environment. MeHg toxicity is characterized by a broad range of molecular cellular effects, often, if not always, induced through the strong affinity MeHg has for thiol groups. Through epidemiological and experimental studies, it has been concluded that dietary status can beneficially affect the severity of MeHg toxicity. However, discrepancies observed in symptomatology after MeHg exposure under different dietary regimes have not yet been elucidated at the molecular level.

In the present study, the molecular interactions between the marine n-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) with MeHg was sought investigated. Additionally, whether each specific n-3 marine fatty acids could affect MeHg toxicity in an ameliorating or detrimental manner, were investigated.

MeHg toxicity in the kidney of Atlantic salmon (*Salmo salar*) was investigated both *in vivo* and *in vitro*, with subsequent investigation of the marine n-3 fatty acids impact on MeHg toxicity *in vitro*. An *in vivo* study was undertaken to explore how MeHg toxicity, at a sub-lethal level, is manifested in the proteome of the Atlantic salmon kidney. For *in vitro* studies, Atlantic Salmon Kidney (ASK) and Human Embryonic Kidney 293 (HEK293) cells were pre-incubated with DHA or EPA, before exposure to MeHg. These cells were subsequently analyzed for accumulation of MeHg, apoptosis, oxidative stress, and transcriptomic and proteomic differential regulation.

MeHg toxicity in the kidney, and in kidney-derived cells, of the Atlantic salmon was characterized by differential regulation of protein markers related to apoptosis, oxidative stress, structural degeneration, disrupted calcium homeostasis, immunological effects, and effects on cell signalling. When cells were pre-incubated with DHA an increase in MeHg-induced apoptosis was observed, and DHA alone also increased the oxidative stress in HEK293 cells. When cells were pre-incubated with EPA, a decrease in apoptosis was observed. Both DHA and EPA affected

several transcriptomic and proteomic markers concomitantly affected by MeHg. These included markers involved in apoptosis, calcium homeostasis, cell signalling, and structural degeneration.

In conclusion, marine n-3 fatty acids affected the toxicity of MeHg. EPA may prove to be remedial, whereas DHA can be potentially augmenting, to the toxicity of MeHg. This finding underlines the importance of considering interaction effects between nutrient and contaminants when assessing the toxicity of environmental toxicants.

Abbreviations

ALA	α-linoleic acid
ANOVA	Analysis of Variance
ARA	Arachidonic acid
ASK	Atlantic Salmon Kidney (cell line)
ATP	Adenosine-triphosphate
BAD	BCL-2 associated death promoter
Bcl2	B-cell lymphoma 2
Bclx	B-cell lymphoma X
COX 2	Cyclooxygenase 2
cPLA2	Cytosolic phospholipase A_2
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEK293	Human embryonic kidney 293 (cell line)
Hpx	Hemopexin like protein
LA	Linoleic acid
LC MS/MS	Liquid chromatography tandem mass spectrometry
MeHg	Methylmercury
MeHgCl	Methylmercury-chloride
pI	Isoelectric point
PI3K/Akt	Phosphoinositide 3-kinase/Akt
PPAR	Peroxisome proliferator-activated protein
PLC	Phospholipase C
real time RT PCR	Real time Reverse Transcriptase Polymerase Chain Reaction
roGFP	Reduction oxidation sensitive green fluorescent protein
ROS	Reactive oxygen species
RYR	Ryanodine Receptor
Sri	Sorcin

List of publications

Paper I

Nøstbakken, O.J., Martin, S.A.M., Cash, P., Torstensen, B.E., Amlund, H., & Olsvik, P.A. (2011): "Dietary methylmercury alters the proteome in Atlantic salmon (*Salmo salar*) kidney". In press. Aquat. Toxicol.

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1. Introduction

1.1 Methylmercury

Methylmercury (MeHg) is a widespread environmental contaminant produced from inorganic mercury (iHg) mainly through biotic methylation in sulphate reducing aquatic microorganisms (Jensen and Jernelov 1969). MeHg, and other mercury (Hg) species, can be found in varying sources such as in the atmosphere, soil, vegetation, freshwater lakes, and oceans (EFSA 2008). In ocean waters, MeHg account for about 10 to 40 % of the total Hg (EFSA 2008), making the aquatic environment and biota, particularly exposed to this species of Hg. The fact that methylation of Hg also occurs almost exclusively in aquatic systems (EFSA 2008), increases the potential for MeHg affecting aquatic organisms far greater than for terrestrial animals. However, MeHg also bioaccumulate and biomagnify up the aquatic food chain (Morel et al. 1998), eventually posing a health risk for predatory terrestrial animals consuming seafood, including humans.

1.1.1 MeHg accumulation and toxicity

The current knowledge on MeHg toxicity in humans is in large parts based on two severe outbreaks of MeHg poisoning; the industrial release of MeHg in Minamata, Japan (SSSGM 1999), and the accidental consumption of MeHg treated grain in Iraq (Bakir et al. 1973). Both these unfortunate epidemics disclosed much knowledge about the toxicity and symptomatology of acute and chronic high level MeHg poisoning. However, MeHg is distributed on a global scale (Boening 2000) and continues to increase due to further anthropogenic contributions (Dietz et al. 2009). Moreover, irreversible toxic effects have been noted at very low doses of MeHg (Murata et al. 2004), prompting scientists to assume that there may not be a lower threshold for MeHg toxicity (Grandjean et al. 2010). Taken together, this exemplifies that the importance of an increased knowledge of MeHg toxicity, also at lower chronic exposure, is prevalent.

Several epidemiological studies have investigated the effects of low level dietary MeHg exposures across the globe, as reviewed by Myers and Davidson (1998), Clarkson and Magos (2006), and Castoldi et al. (2008). These epidemiological studies have mainly focused on toxicity in the developing nervous systems, which have proven to be particularly sensitive to MeHg toxicity (Aschner and Syversen 2005). MeHg has the ability to cross the placenta barrier, and can also be transfered through breast milk and food (Clarkson and Magos 2006), making MeHg available for uptake during fetal development and in early life stages.

Dietary MeHg is absorbed in the intestine with an efficiency of 90-95% and is, in vertebrates, distributed throughout the organism by the vascular system (Sanfeliu et al. 2003). MeHg exert its toxic effects in different tissues, but is generally regarded as a neurotoxic compound due to the severe effects observed in mental faculties. However, accumulation of MeHg seem to occur throughout organisms, though in mammals the highest concentrations are observed in kidney and liver (Rodrigues et al. 2010).

Demethylation of MeHg has been reported to occur in phagocytic cells of different species (Suda et al. 1992), and in liver microsomes in rats (Suda and Hirayama 1992). iHg is not as toxic as MeHg, and also displays different toxicological characteristics. The kidney is particularly prone to accumulation of iHg. Taken together, this could mean that the toxicity displayed in kidney after MeHg exposure, is a kind of mixture toxicology between iHg and MeHg. However, accumulation studies have revealed that the bulk part of Hg in kidney exists as MeHg in rats exposed to dietary MeHg (Rodrigues et al. 2010).

A broad range of MeHg induced neurological symptoms have been reported, ranging from mild sensory impairments to severe deterioration of cognitive functions, paralysis and death (Sanfeliu et al. 2003). However, MeHg toxicity is not limited to the neurological system. Detrimental effects of MeHg have been reported also in the kidney, liver, cardiovascular system, immune system, and in the blood components (ATSDR 1999; CTM 2001)

1.1.2 Molecular toxicity of MeHg

MeHg has a high affinity for thiol groups (Hughes 1957), and several toxic effects of MeHg have been sought understood in light of this molecular characteristic (Sanfeliu et al. 2003). Due to the strong affinity MeHg has for the sulfur containing amino acids cysteine and methionine, proteins can mediate the toxic effect of MeHg through for example, inhibition of enzymatic activities, degradation of structural components such as microtubule, and through affecting phosphorylation pathways. (Usuki et al. 2008; Crespo-López et al. 2009; Franco et al. 2009). Although much of the toxicity of MeHg can be explained through its interactions with thiols, knowledge on how MeHg-thiol binding is linked to all aspects of MeHg toxicity is not yet fully understood (Sanfeliu et al. 2003). In a review by Ceccatelli et al. (2010), MeHg thiol-binding capability and subsequent toxicity was listed together with MeHg-induced effects on calcium (Ca^{2+}) homeostasis and induction of oxidative stress as a mechanism of toxicity. However, thiol-binding and secondary cellular effects such as oxidative stress can in some cases be explained together through causality. As an example, MeHg induction of oxidative stress can be explained at least partly through its thiol binding capabilities, given that binding of MeHg to the thiol-containing glutathione deplete the cell of glutathione, inhibiting the innate antioxidant system of the cells and thereby disturbing the red-ox environment, creating reactive oxygen species (ROS) (Dreiem et al. 2005). To distinguish between molecular mechanisms of MeHg toxicity, such as thiol binding, and the secondary manifestation of toxicity such as induced oxidative stress, an increased knowledge of intracellular systemic molecular mechanisms is of vital importance in order to fully understand the complete toxicological repercussions of MeHg toxicity.

Although thiol binding capability may be the biochemical rationale behind MeHg toxicity, several cellular effects, or “secondary manifestations” of toxicity, have been reported in the vast literature that covers MeHg toxicity (Table 1).

Table 1. Cellular effects caused by methylmercury.

Cellular effects	Reference
Oxidative stress	(Farina et al. 2011)
Disruption of intracellular Ca^{2+} homeostasis	(Ceccatelli et al. 2010)
Disruption of oxidative phosphorylation	(Bourdineaud et al. 2009)
Cell signaling	(Chen et al. 2006; Usuki et al. 2008; Fujimura et al. 2009)
Ubiquitin proteasomal degradation	(Hwang 2007)
Genotoxicity/Carcinogenesis	(Boffetta et al. 1993; Crespo-Lopez et al. 2007; Crespo-López et al. 2009)
Apoptosis	(Ceccatelli et al. 2010; Cuello et al. 2010)
Disruption of microtubules	(Sager et al. 1982)
Immunological effects	(Gardner et al. 2010)

When reviewing the experimental data published on MeHg toxicity, it becomes evident that MeHg is a compound with many modes of action, and that assessing the importance of all molecular mechanisms can be tedious. Still, certain molecular mechanism behind its toxicity seems to be more prevalently mentioned in the literature than others, e.g. induction of ROS, perturbation of intracellular Ca^{2+} levels, and disruption of cytoskeletal components, in particular microtubule.

1.1.3 MeHg in fish

MeHg can be toxic to fish both at population level (Scheuhammer et al. 2007), as well as under controlled experimental conditions in individual fish (Berntssen et al. 2003). Obviously, there exists great diversity in fish species, and consequently great interspecies variability in response to MeHg toxicity. However, due to the relatively limited studies on MeHg toxicity in Atlantic salmon, collective results from MeHg

related research in different teleosts will be discussed below with emphasis on the salmonid species.

Uptake of total Hg in freshwater fish has been shown to be dependent on pH and hardness of the water, as reviewed by Boening (2000). Most of the total Hg in Atlantic cod (*Gadus morhua*) occurs as MeHg (Bloom 1992), suggesting that accumulation also is dependent on the extent of Hg-methylation in the local environment. Accumulation of total Hg in Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) is comparatively highest in the blood, liver, and kidney, with a smaller amount accumulated in the putative MeHg-target organ, namely the brain (Giblin and Massaro 1973; Boudou and Ribeyre 1985; Berntssen et al. 2003). Elimination of total Hg from Atlantic cod is slow (Amlund et al. 2007), particularly in the muscle (Giblin and Massaro 1973), thereby making bioaccumulation in fish a crucial issue when addressing MeHg transfer both within aquatic systems, and further to higher order mammals.

Symptoms of MeHg toxicity similar to that in mammals have also been observed in fish. The ability of MeHg to reduce the response of the olfactory system of the Atlantic salmon (Baatrup et al. 1990), suggests that the neurological system is a target organ for MeHg also in Atlantic salmon. More direct observed pathological changes in neurological tissue, such as severe vacuolation and necrosis have also been observed (Berntssen et al. 2003). However, MeHg toxicity is not limited to the neurological system. Lock and van Overbeeke (1981) showed that MeHg, through water exposure, also affect the mucus excretion on the skin and gills of rainbow trout which can cause respiratory problems for the fish. An impact of MeHg on fish reproduction have been suggested in a review by Scheuhammer et al. (2007). This is supported by studies in fathead minnow (*Pimephales promelas*) reporting MeHg-induced interference in gonadotropin pathways (Klaper et al. 2008), and MeHg-induced increase of apoptotic follicular cells in ovarian follicles (Drevnick et al. 2006), suggesting decreased fecundity.

Exploratory studies using transcriptomics or proteomics have investigated the molecular toxicity of MeHg in several different fish species such as zebrafish (*Danio rerio*) (Gonzalez et al. 2005; Richter et al. 2011), fathead minnow (Klaper et al. 2008), beluga (*Huso huso*) (Keyvanshokooh et al. 2009), and Atlantic cod (Berg et al. 2010). When different types of tools for functional annotation and pathway analysis were implemented, the most frequently reported molecular mechanisms in response to MeHg are apoptosis, oxidative stress and pernicious effects on energy metabolism (Klaper et al. 2008; Berg et al. 2010; Richter et al. 2011), These findings are consistent with findings in mammalian studies (Ceccatelli et al. 2010). The occurrence of apoptosis has in several cases been linked to the increased influx of Ca^{2+} into the cells, based on the regulation of several Ca^{2+} regulating and dependent proteins (Keyvanshokooh et al. 2009; Berg et al. 2010). Oxidative stress caused by MeHg in fish have been attributed to its sulfhydryl-binding capabilities, and hence its depletion of glutathione in cells which is reflected in differential expression of biomarkers relating to the antioxidant systems (Berntssen et al. 2003; Berg et al. 2010; Richter et al. 2011). Detrimental effects of MeHg on energy metabolism have been particularly attributed to mitochondrial disruption and regulation of markers related to glycolysis (Berg et al. 2010; Richter et al. 2011).

1.2 Marine n-3 fatty acids

N-3 fatty acids are a group of long chain polyunsaturated fatty acids with the first double bond at carbon number three counting from the methyl end. Based on the source of origin docosapentaenoic acid (DPA, 22:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and eicosapentaenoic acid (EPA, 20:5n-3), are also defined as marine n-3 fatty acids. The essential fatty acids linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) cannot be synthesized in humans or fish (Spector 1999), and function as precursors for EPA, DHA, and arachidonic acid (ARA, 20:4n-6) which are all necessary for good health. The marine fatty acids can to a certain degree be synthesized in humans from ALA, however this synthesis is very limited and the marine n-3 fatty acids DHA and EPA are considered to be conditionally essential in humans (Calder et al. 2010). In Atlantic salmon, the traditional diets consist of high amounts of DHA and EPA and consequently their ability to synthesize these unsaturated fatty acids are, similarly to in mammals, low (Leaver et al. 2008). In humans, the marine n-3 fatty acids EPA and DHA are important constituents of a balanced diet to obtain optimal growth and development, and to avoid later onset of chronic disease (Calder et al. 2010). Beneficial effects of EPA and DHA have been observed in preventing cardiovascular disease, reduce inflammation, and for optimal neuronal development (Uauy et al. 2001; Breslow 2006; Calder 2006). To a lesser degree, also possible negative health impacts of marine n-3 fatty acids have been reported, such as increased lipid peroxidation, prolonged bleeding time, gastrointestinal disturbance and impaired lipid and glucose metabolism (VKM 2011). However, the adverse effects of marine n-3 fatty acids are linked to very high concentrations, and the beneficial effects of the marine n-3 fatty acids seem to clearly outweigh the adverse effects (ibid). In the aquaculture of Atlantic salmon, feed production has been steered towards the use of plant based raw materials due to limited access of marine raw materials (Torstensen et al. 2008). Consequently, the amount of DHA and EPA in fish tissues decreases (Torstensen et al. 2005). Still, studies have shown that decreased amount of DHA and EPA in feed for Atlantic

salmon have not increased mortality or decreased the growth rate of the fish, but will alter the lipid composition of different fish tissues (Turchini et al. 2009).

1.2.1 Molecular mechanisms of marine n-3 fatty acids

Dietary marine n-3 fatty acids are taken up in the intestine and distributed through the vascular system bound to lipoproteins or albumin. Fatty acids are taken up in tissue through active protein mediated, or through passive diffusion processes (Su and Abumrad 2009; Zhou et al. 2010), and are further incorporated into the membrane or taken up into cytosol of cells. Marine n-3 fatty acids which are incorporated in the membrane can influence its physical properties such as organization, ion permeability, elasticity and microdomain formation (Gorjao et al. 2009). In particular DHA constitutes a large part of neurological cell membranes, and deficiency in DHA cause deficits in neuronal development (Innis 2008; Newland et al. 2008). EPA has also shown to be an important agent in treating mental illness in humans (Hibbeln 1998; Appleton et al. 2006) although mechanisms explaining these effects are more elusive.

Twenty carbon fatty acids, such as EPA, can mediate its effects through the eicosanoid system (Spector 1999). In this molecular pathway, the fatty acids are cleaved from phospholipids at the sn2 position by cytosolic phospholipase A2 ($cPLA_2$), and are released for further processing by cyclooxygenases, lipoxygenases or cytochrome P450s (Figure 1).

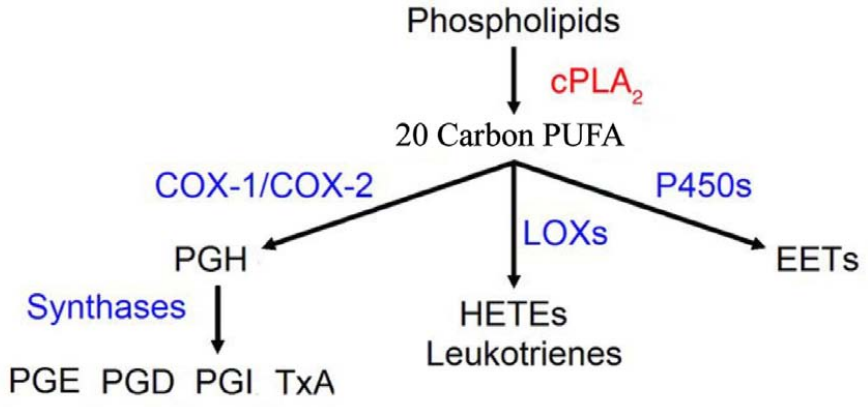

Figure 1. The eicosanoid pathway converting 20C polyunsaturated fatty acids into different active metabolites. Figure modified from Adler et al. (2008). Abbreviations: $cPLA_2$ cytosolic phospholipase A_2, P450s cytochrome P450s, COX1 and 2 cyclooxygenase 1 and 2, PGH prostaglandin H, LOXs lipooxygenases, PGE Prostaglandin E, PGD prostaglandin D, PGI prostaglandin I, TxA Thromboxane A, HETEs hydroxyeicosatetraenoic acid, EET epoxyeicosatrienoic acid.

Compared to the n-6 fatty acid ARA, which is the prevalent substrate for this pathway, EPA display different effects in the cells due to different metabolites produced (Bagga et al. 2003). For example, prostaglandin E3, which is produced from EPA through cyclooxygenase 2 (COX2), has been shown to have a significantly lower inflammatory effect than the ARA derived equivalent Prostaglandin E2 (Bagga et al. 2003). Additionally to the eicosanoid signalling pathway, marine n-3 fatty acids have been shown to be involved in several other signalling pathways such as the phosphoinositide 3-kinase/Akt (PI3K/Akt) (Wan et al. 2007), the janus kinase/signal transduction and transcription (JAK/STAT) (Gorjao et al. 2007) and the extracellular-signal-regulated kinase (ERK) pathway (Denys et al. 2001). The fact that marine n-3 fatty acids exert their effects through several signalling pathways, increase the complexity when trying to elucidate molecular mechanisms behind the beneficial effects of these fatty acids.

Catabolism of EPA occurs through mitochondrial β-oxidation, whilst DHA have to undergo the less efficient oxidation in peroxisomes (Braiterman et al. 1999). These metabolic pathways differ, for example the net energy gain from peroxisomal

compared to mitochondrial β-oxidation is halved (Mannaerts and van Veldhoven 1996). In addition, peroxisomal β-oxidation of fatty acids does not go to completion (Lazarow 1978), but acts as a chain shortening system, before remnants of the fatty acid is metabolized further in the mitochondria. Catabolism of fatty acids is an important addition to the cells energy demands, however, different routes of catabolism may result in different outcomes for the cell. Since peroxisomal β-oxidation is not coupled to ATP production, but instead yields H_2O_2, the production of ROS may be a detrimental side effect of peroxisomal catabolism (Schrader and Fahimi 2006).

Marine n-3 fatty acids have a great therapeutic effect on cardiovascular diseases, particularly due to their suppression of arrhythmia (Breslow 2006). This effect has partly been attributed to the marine n-3 fatty acids regulatory effects on Ca^{2+} homeostasis (Siddiqui et al. 2008). DHA and EPA have been shown to modulate Ca^{2+} uptake through the L-type voltage gated channel, and to inhibit intracellular Ca^{2+} release through the ryanodine receptor (RyR) channel in the sarcoplasmic reticulum (Swan et al. 2003). Additionally, EPA has been shown to ameliorate the effects of the cardiac toxin ouabain, which blocks the Na^+/K^+-ATPase and subsequently disturbs the homeostasis of Ca^{2+} (Hallaq et al. 1990). This ameliorating effect of EPA occurs through modulation of intracellular Ca^{2+}, possibly through direct binding to the sodium ion channel (Siddiqui et al. 2008).

1.3 Interaction between marine n-3 fatty acids and MeHg

MeHg is one of the best described toxicants in the literature with over a thousand publications in experimental toxicology (Grandjean et al. 2010). Still, the molecular mechanisms responsible for its detrimental effects are not clear (Sanfeliu et al. 2003; Clarkson and Magos 2006). Indications of nutrition having a major role in MeHg toxicity calls for further studies on nutritional effects on MeHg (Chapman and Chan 2000; Sanfeliu et al. 2003; Clarkson and Magos 2006; Stokes-Riner et al. 2011). In addition, when studying modulating effects of nutrients on MeHg toxicity, a clearer picture on how MeHg exerts its molecular toxicity may emerge due to a better

understanding of the interactions observed. The ability of different organisms to deal with toxic insults may also be dependent on the quantity and quality of their diet (Archer et al. 2001). Understanding how nutrients and MeHg interact will therefore be valuable both in terms of understanding the molecular toxicology, and for ensuring proper dietary advisory (Hennig et al. 2004).

1.3.1 Epidemiological studies

Several epidemiological studies have been performed during the last 40 years, where low chronic doses of MeHg were investigated (Grandjean et al. 2010). From these, three main studies investigating the neurodevelopmental effects of MeHg have gained most attention due to the magnitude of these studies (Clarkson and Magos 2006). These are the New Zeeland study (Crump et al. 1998), the Faroe Islands study (Grandjean et al. 1997), and the Seychelle island study (Myers et al. 2003). In both the Faroese and New Zealand study, delayed brain development and cognitive deficits were observed (Grandjean et al. 2010). However, in the study performed at the Seychelles no detrimental effects of MeHg were observed (Myers et al. 2003). This has led researchers to the notion that there could be certain nutrients in the Seychelle diet which possibly ameliorated or masked the MeHg toxicity (Myers and Davidson 1998). In more recent research, Strain et al (2008) observed that if both marine n-3 fatty acids and MeHg were adjusted for in the statistical analysis, MeHg adversely affected, while marine n-3 fatty acids benefitted neuronal development. However this effect was diminished if the two factors were analyzed separately (Strain et al. 2008), suggesting that marine n-3 fatty acids counteract MeHg toxicity. Beneficial dietary effects of fish consumption versus the risk of MeHg exposure have been assessed in several epidemiological investigations and reviews (Stern and Korn 2011; Ström et al. 2011). Teutsch and Cohen (2005) have also underlined the importance of proper guidelines for policy makers in making dietary advisory. Still the literature addressing the mechanisms behind molecular interactions between marine n-3 fatty acids and MeHg remain scarce.

1.3.2 Molecular mechanisms of interactions

Epidemiological studies strongly suggest a link between marine n-3 fatty acids and consequent decreased MeHg toxicity. However, the mechanisms behind these interactions have not yet been elucidated. Interpretation of epidemiological studies may infer that the basis for ameliorating effects observed is simply that nutrients cover up of MeHg detrimental effects, since marine n-3 fatty acids improve the same parameters measured. Strikingly, the marine n-3 fatty acids affect in a beneficial manner similar functions which MeHg affect in a more detrimental manner, particularly within neuronal development and cardio-vascular health. More specific molecular mechanisms also tend to be oppositely affected by marine n-3 fatty acids and MeHg. Marine n-3 fatty acids have been reported to regulate Ca^{2+} homeostasis (Siddiqui et al. 2008) while MeHg exerts its toxicity through disruption of Ca^{2+} homeostasis (Ceccatelli et al. 2010). Moreover, marine n-3 fatty acids have been reported to modulate the red-ox environment in cells (Shimazawa et al. 2009; Østbye et al. 2011), which also is a known detrimental effect of MeHg (Farina et al. 2011). Additionally, marine n-3 fatty acids can affect cell metabolism both in a regulatory manner through the nuclear receptors and transcription factors peroxisome proliferator receptor (PPAR) and liver X receptor (LXR) (Li and Glass 2004), but also by acting as a substrate for catabolism. In line with this, Chapman and Chan (2000) ascertain that interaction studies between nutrients and MeHg fall into two categories, that is, effects of nutrients on Hg metabolism, and effects of Hg on nutrient metabolism. Several studies have reported that MeHg affect metabolism, both *in vivo* (Thukral et al. 2005; Bourdineaud et al. 2009; Berg et al. 2010) and *in vitro* (Yee and Choi 1996). These studies clearly indicate a detrimental effect of MeHg on nutrient energy metabolism in the cell, particularly the detrimental effect on mitochondrial glycolysis and oxidative phosphorylation has been emphasized (Bourdineaud et al. 2009; Berg et al. 2010). However, the effect of nutrients, and specifically marine n-3 fatty acids, on MeHg metabolism is much less understood.

Several studies have investigated the possible ameliorating effect of fish fillet, fish oil or DHA as a supplement, on behavioural effects, accumulation and neurotoxicity of

MeHg *in vivo* without finding any clear causality (Day et al. 2005; Paletz et al. 2007; Bourdineaud et al. 2011). Contrary to this, there also exists both *in vitro* and *in vivo* studies showing interaction effect of fish-derived supplements on MeHg toxicity (Kaur et al. 2007; 2008; Jin et al. 2009; Grotto et al. 2011; Jayashankar 2011).

In mice pups fed diets spiked with DHA a decreased accumulation of MeHg in brain, but not in liver, was observed (Jayashankar 2011). However, other *in vivo* studies have observed no difference in the accumulation and distribution of MeHg (Day et al. 2005; Grotto et al. 2011). *In vitro* studies using both commercially available neuronal cell lines and mice primary neuronal cells, have also shown that DHA can decrease the uptake of MeHg (Kaur et al. 2007; 2008).

Oxidative stress has been regarded as one of the most important toxic mechanisms induced by MeHg (Farina et al. 2011). Modulation of oxidative stress by fish oil or marine n-3 fatty acids has also been assessed. Grotto et al. (2011) showed no preventive effects of fish oil on MeHg induced oxidative stress markers in rats. Additionally Kaur et al (2007; 2008) observed an increase in the generation of ROS and increased depletion of glutathione after pre-incubating cells with DHA before MeHg exposure in primary cerebellar neurons, C6 glial cells, and B35 neuronal cells. However, an attenuation of oxidative stress was observed in primary astrocytes as a result of DHA pre-incubation and MeHg exposure (Kaur et al. 2008). DHA alone has also been shown to induce ROS generation by itself in these studies.

In addition to investigating MeHg-distribution and oxidative markers, Grotto et al. (2011) also observed an attenuating effect of fish oil on DNA damage caused by MeHg. Moreover, in rats fed fish oil a decrease in leucocyte infiltration in different tissues was observed, suggesting an anti-inflammatory effect of fish oil (Grotto et al. 2011).

2. Aims of the study

This study was designated to investigate if marine n-3 fatty acids affect MeHg toxicity in Atlantic salmon, and if so, how this is manifested on a molecular level.

Specific aims of this study were to:

- ✓ Explore *in vivo* MeHg toxicity in kidney of Atlantic salmon
- ✓ Investigate possible modulation of MeHg toxicity *in vitro* by the n-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).
- ✓ Explore molecular mechanisms behind fatty acid modulation of MeHg toxicity.

3. Summary of results

3.1 MeHg toxicity in the kidney of Atlantic salmon

Paper I reports on the toxicity of dietary MeHg in the Atlantic salmon kidney through the investigation of MeHg accumulation, expression studies by means of real time RT-PCR, and proteomic studies.

Fish exposed to dietary MeHg accumulated significantly more Hg than control fish, particularly in liver, kidney and red blood cells. The proteomic analysis revealed differential abundance of twenty-six spots in the kidney, and fourteen of these protein spots were successfully identified. The proteins identified indicated effects of MeHg on metabolism, immunological markers, oxidative stress, protein-folding, and cell structure. No transcriptional differential regulation was observed in the kidney in response to MeHg. The results indicated that, besides the immunological markers differentially regulated, dietary MeHg display many similar effects in the kidney as previously shown for other tissues in fish.

3.2 *In vitro* effects of DHA and EPA on MeHg toxicity

Paper II describes the modulation of MeHg toxicity through marine fatty acid pre-incubation in the Atlantic salmon cell line Atlantic Salmon Kidney (ASK), and the human cell line Human Embryonic Kidney 293 (HEK293) cells.

ASK and HEK293 cells were pre-incubated with DHA, EPA, or ARA prior to MeHg-exposure, and cell toxicity was assessed by measuring MeHg-uptake in cells, proliferation, apoptosis, oxidative stress, and regulation of selected toxicological and metabolic transcriptional markers. DHA decreased the uptake of MeHg in HEK293 and increased MeHg-induced apoptosis in ASK cells; while EPA decreased apoptosis in ASK. MeHg exposure induced changes in selected metabolic and known MeHg toxicological biomarkers. Both DHA and MeHg alone, but not EPA, induced a change in the oxidative status of HEK293 cells.

3.3 Molecular mechanisms of DHA and EPA on MeHg toxicity in ASK cells.

Paper III reports the differential expression of the proteome in response to DHA and EPA pre-incubation and subsequent MeHg exposure in ASK cells.

ASK cells were pre-incubated with the marine n-3 fatty acids DHA and EPA before exposure to MeHg. Modulating effects of the marine fatty acids on MeHg toxicity were subsequently assessed using the exploratory technique of proteomics in a factorial design. Thirty-four differentially regulated proteins were identified. From these, twenty-seven were shown to be differentially regulated by MeHg, twelve were regulated by the fatty acids, and another eight showed interaction effects between MeHg and the fatty acids. Functional annotations and pathway analysis of the proteins revealed that marine n-3 fatty acids and MeHg concurrently affected the proteomic abundance of markers relating to such molecular mechanisms as cell signaling, Ca^{2+} homeostasis, structural integrity, apoptosis, and energy metabolism.

4. General discussion

This study was designated to investigate molecular effects of MeHg and marine n-3 fatty acids in Atlantic salmon. An understanding of how marine n-3 fatty acids and MeHg interact in Atlantic salmon would provide beneficial background information for follow-up research investigating the effects in mammals fed Atlantic salmon containing MeHg.

4.1.1 Atlantic Salmon (*Salmo salar*)

Atlantic salmon farming makes up the bulk part of the Norwegian aquaculture industry and is important both in economic terms and as a nutritional contribution to a healthy balanced diet. Still, the Atlantic salmon is a carnivorous fish where traditionally a large part of its feed has been derived from marine sources, such as fish meal and oil produced from relatively high trophic layer pelagic species, which can contain MeHg in the protein fraction. Due to an increase in the global pollution of Hg (Dietz et al. 2009), the total Hg content in fish-feeds based on pelagic species could be increasing. However, fish meal and fish oil are increasingly being replaced by alternative feed ingredients (Torstensen et al. 2008) which cause a decrease in dietary MeHg to farmed Atlantic salmon concomitant with the decrease in the amount of marine fatty acids in fish tissues (Torstensen et al. 2005). This creates a new backdrop for MeHg risk-benefit analyses of Atlantic salmon feed and fillet, both for fish health evaluations but also for transfer of contaminants to consumers. An improved understanding of how nutrients and contaminants interact would substantiate a beneficial knowledge for such risk-benefits analyses.

The rationale for using Atlantic salmon was to investigate MeHg and marine n-3 fatty acids interaction in a fish cell system. Still, general mechanisms may be extrapolated from salmonid systems to mammalian systems, making Atlantic salmon a model species. Except for the higher cognitive neurological effects, similar effects of MeHg have been observed in Atlantic salmon as well as in humans.

4.1.2 The kidney as a MeHg-target organ

The neurological system is regarded as the main toxicological target organ for MeHg (Berg et al. 2010; Ceccatelli et al. 2010), but distribution and toxicological impact of MeHg is not limited to the brain, neither in fish nor mammals (Berntssen et al. 2003; Rodrigues et al. 2010). The kidney, both in mammals and fish, accumulate among the highest amounts of MeHg in the organisms (Boudou and Ribeyre 1985; Rodrigues et al. 2010), and nephro-toxicological effects have been observed in both rat and Atlantic salmon kidney (Berntssen et al. 2003; Jin et al. 2009). The toxicity of MeHg in kidney is generally not well known, and MeHg toxicity in kidney from Atlantic salmon is basically unknown.

4.1.3 Use of cell culture, *in vitro* versus *in vivo*

In this thesis, both *in vivo* and *in vitro* studies have been implemented to obtain as extensive view of MeHg toxicity in the Atlantic salmon kidney as possible. Still, the main mechanism studies have been performed in cells. Cell culture is a powerful tool for obtaining mechanistic understanding of toxicology (Zucco et al. 2004). However, to do *in vitro* studies also means to appreciate the limitations of the system studied, and therefore an *in vivo* study of MeHg toxicity in Atlantic salmon was included for comparative reasons.

The main aim of the study was to investigate the mechanistic interplay between marine n-3 fatty acids and MeHg in a Atlantic salmon cell system. The MeHg-induced effects on the proliferation of cells, and the effects of relative low concentration of MeHg over a longer time period were aimed to simulate an *in vivo* long term chronic exposure. This abolished the choice of using primary cells which are not proliferating and have a short window of survival. Atlantic Salmon Kidney (ASK) cells, a stable cell line developed by Devold et al. (2000), were chosen. These cells proliferate, they occur in monolayer, and are derived from Atltantic salmon kidney, making them suitable for this study. To further elucidate the effects of marine n-3 fatty acids and MeHg, the mammalian kidney cell line (HEK293) was included to gain broader insight into possible molecular mechanisms. Additionally, HEK293

cells grow faster, and are more easily manipulated by molecular techniques such as e.g. transfection techniques, making these cells a valuable additional tool and mammalian counterpart in our studies.

4.1.4 The exploratory experimental design

To my knowledge, the manner in which MeHg toxicity affects the Atlantic salmon kidney is largely unknown. Additionally, the effect of the individual marine n-3 fatty acids on MeHg toxicity is described to a minor degree. With this in mind, an exploratory approach was undertaken to elucidate our aims. To best describe possible interaction effects, factorial designs were used as the main experimental design as to increase the control over variation and thereby increase the statistical power (Festing 2004).

To assess MeHg toxicity and the possible modulating effects of marine n-3 fatty acids proteomic analyses were implemented, or more specific, profiling-proteomic analyses (Figeys 2002). In profiling proteomics, the proteome of cells or tissue are separated on a 2D gel with subsequent identification of protein spots using different kinds of mass spectrometry (MS) methods (ibid). By using pre-cast gels, separation of proteins is more reproducible than by use of "homemade" gels. However, proteomics does not pick up all differentially regulated proteins in an organ or cell extract. Some proteins will be expressed below detection limit of the gel, while other will not be properly separated due to the limits chosen for each gel. Gels with PI limit of 4-7 and mass limit of 14-191kDa were used (Paper I and III), and proteins which fall short of these boundaries will not be detected. There are also issues regarding the sample preparation where certain proteins will not be included, for example due to high lipophilicity and incorporation in membrane, certain proteins will be removed in early centrifugation steps. Despite the fact that proteomics does not pick up every protein, it is a high-throughput exploratory method which can generate clues as to what proteins are responsible for the mechanisms observed in this study. It can also generate working hypothesis for later analysis, which can be verified using specific real time RT PCR or western blot.

In order to extract as much information as possible from the proteomic results, the bioinformatic tool of Ingenuity Pathway Analysis (IPA) was implemented (Paper I and III). This tool relates the differentially regulated, and annotated, proteins observed, to its large databank of already known proteins and their function. It further generates interaction networks and gives information about how the proteins can affect cellular function, diseases, and molecular pathways (Paper III), illustrating complex data sets of expressed proteins interpreted at a functional level possibly improving the result interpretation and conclusions.

4.2 MeHg toxicity

MeHg toxicity was investigated in kidney of Atlantic salmon, both *in vivo* (Paper I) and *in vitro* (Paper II and III). After MeHg exposure *in vivo*, a high accumulation of Hg was observed in kidney, liver and red blood cells (Paper I), which is consistent with previous studies in Atlantic salmon and rainbow trout (Boudou and Ribeyre 1985; Berntssen et al. 2003). However, no toxic effects of MeHg were observed in the Atlantic salmon systemic physiological parameters: mortality, weight, length or haematocrit, after eight weeks of exposure (Paper I). MeHg toxicity was further assessed using real time RT-PCR detection of selected markers, proteomics, and different *in vitro* investigations. Through these investigational strategies several sub-lethal cellular effects of MeHg toxicity was encountered (Figure 2).

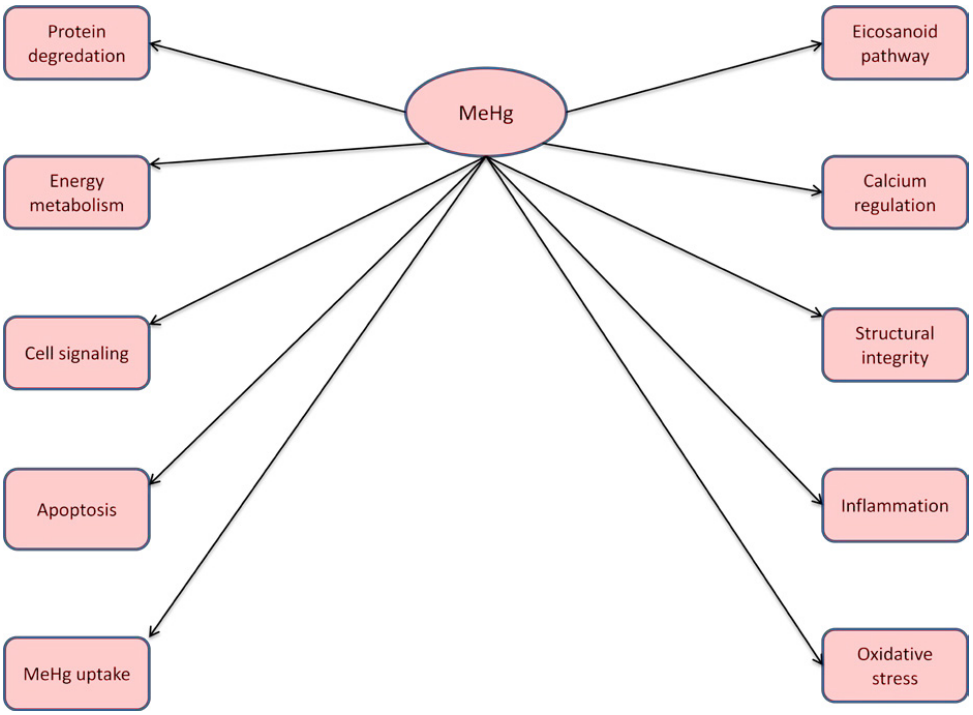

Figure 2. Overview of cellular effects of methylmercury in the kidney of Atlantic salmon in vitro and in vivo, observed in our study.

MeHg toxicity display certain *in vitro* characteristics such as increased apoptosis, structural degeneration, increased oxidative stress, disrupted Ca^{2+} homeostasis, altered cell signalling, and effects on energy metabolism. Due to key regulatory effects observed from the fatty acid pre-incubation (Paper II and III), apoptosis, oxidative stress, Ca^{2+} homeostasis, and cell signalling will be discussed in the later sections.

One of the most prominent effects of MeHg observed throughout this study, both *in vitro* and *in vivo*, was its ability to affect cell morphology and cell structural components. Members of the actin filament, the intermediate filament, and the microtubule filament, along with several anchoring proteins, were differentially regulated in response to MeHg (Paper I, II and III). The microtubule filament is a dynamic filament continually depolymerising and assembling in cells (Burchill et al. 1978). MeHg has been shown to directly bind to cysteines within the tubulin dimers, thereby inhibiting its assembly (Vogel et al. 1985), and making the thiol affinity of MeHg responsible for microtubule disruption. Direct binding to cysteines may also be part of the explanation of differential regulation of actin filament related proteins. However, structural degeneration may also be secondary to other cellular effects caused by MeHg. Apoptosis, for example, is a controlled demolition of the cell resulting in major structural reconfiguration of the cells (Elmore 2007), which was visible in the microscope (Figure 3). Apoptosis can consequently explain the many effects observed in proteins and transcripts involved in the actin filament build-up (Paper I and III). Additionally, several of the structural related proteins observed are also regulated directly or indirectly through Ca^{2+} dependent-mechanisms, which imply that a disruption of Ca^{2+} homeostasis can ultimately affect regulation of these proteins. MeHg and fatty acid effects on Ca^{2+} homeostasis and apoptosis will be discussed below.

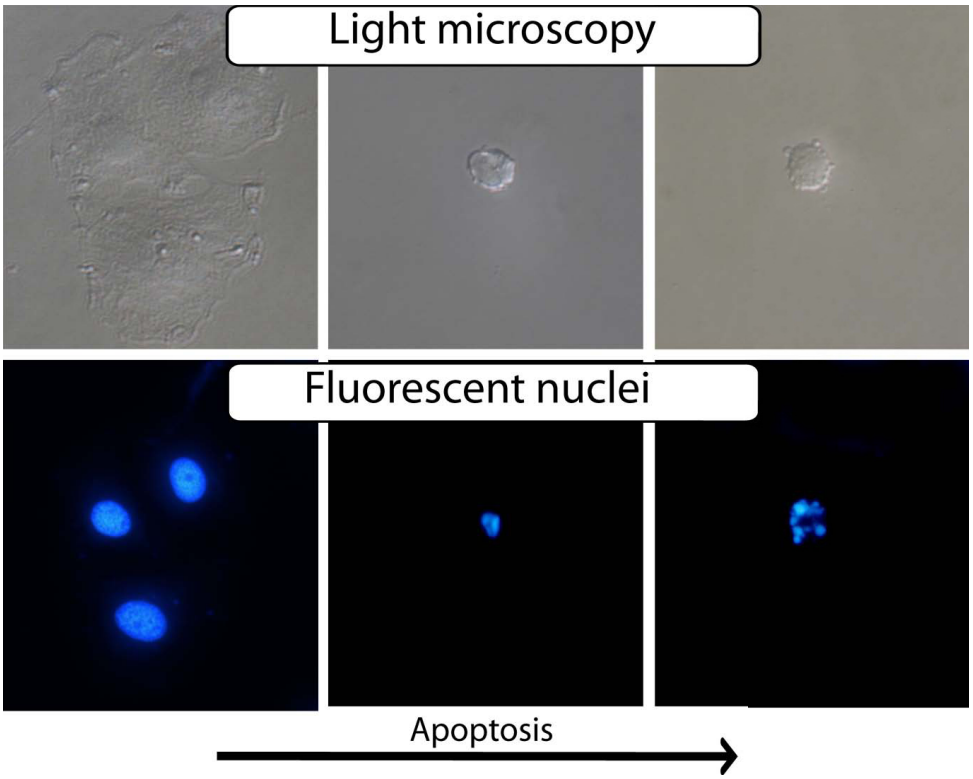

Figure 3 ASK cells undergoes severe morphological changes during apoptosis. The early apoptotic process is characterized by condensing of the nucleus (pyknosis) and cell shrinkage. Subsequently, the plasma membrane starts to show signs of blebbing, and the nucleus is fragmented (karyorrhexis). Eventually, the cells fragment, and are (in vivo) phagocytised by phagocytic cells. In vitro, however, there are no phagocytizing cells, and the apoptotic cells eventually bursts.

Several proteomic markers related to the ubiquitin-proteasomal degradation of proteins were identified in this study (Paper III). The proteins ubiquilin 4 (UBQLN4), suppressor of G2 allele of SKP1 homolog (SUGT1), and proteasome inhibitor PI31 subunit (PMSF1) all showed an increase in abundance in response to MeHg (Paper III). Similarly to microtubule disruption, increased protein degradation can be attributed to the ability of MeHg to bind strongly to cysteines in proteins, disrupting their conformation and function, eventually leading to increased protein degradation. Hwang et al. (2002) showed that by inhibiting ubiquitin-proteosomal degradation in cells, MeHg toxicity increased. Additionally, in a mutant cell line which cannot degrade ubiquitinated proteins, hypersensitivity towards MeHg were shown, both

suggesting a cyto-protective role for the ubiquitin-proteasomal system during MeHg exposure (Hwang et al. 2002).

To further elucidate the molecular mechanisms of MeHg, and to elucidate modulating mechanisms, an investigation into how the marine n-3 fatty acids DHA and EPA can affect MeHg toxicity *in vitro*, were undertaken. Therefore, MeHg toxic mechanisms will be discussed in the context of how marine fatty acids might alter the specific MeHg molecular toxicity.

4.3 Interactions between n-3 fatty acids and MeHg.

The marine n-3 fatty acids DHA and EPA modulated, in different ways, several of the molecular effects displayed by MeHg (Figure 4).

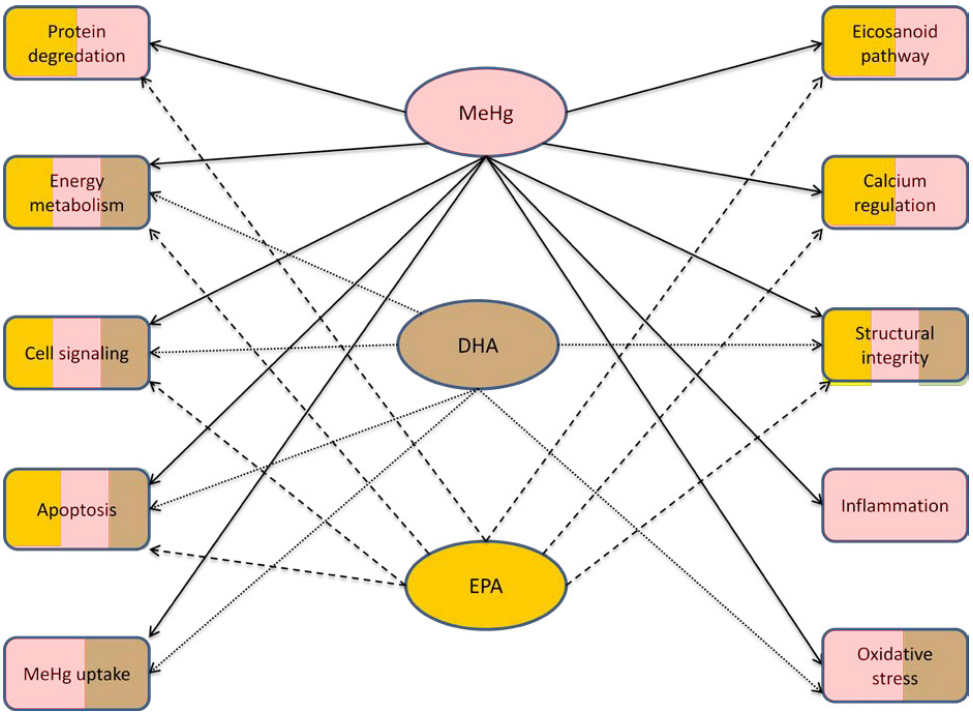

Figure 4. Depiction of how interactions, and concomitant effects, between marine n-3 fatty acids and MeHg were observed in the same cellular mechanisms in our study.

In addition to marine n-3 fatty acids modulating effects on MeHg metabolism, MeHg may also affect the metabolism of nutrients, such as the marine n-3 fatty acids. MeHg detrimentally affect several aspects of cell energy metabolism (Paper I, II, and III) (Bourdineaud et al. 2009; Berg et al. 2010). Fatty acid transport protein 1 (*fatp1*) was used as a transcriptional indicator for the cells fatty acid uptake, and carnitine palmitoyltransferase 1 (*cpt1*) for mitochondrial fatty acids uptake. Both these transcriptional markers were significantly down-regulated in response to MeHg thereby decreasing the availability of fatty acids as energy substrate for the cells (Paper II). Moreover, the abundance of protein-markers relating to glucose

metabolism such as for glycolysis, glyceraldehyde-dehydrogenase (Gapdh) and for pentose pathway, transketolase-like protein 2 (Tktl2), were shown to be down-regulated by MeHg (Paper I and III), suggesting a decrease also in glucose metabolism. As regulation of apoptosis has been clearly linked to energy metabolism (Majors et al. 2007), a decrease in efficient energy metabolism in the cell may speed up the apoptotic process. Under normal circumstances, there is a consistent presence of energy in the form of ATP in cells undergoing apoptosis, but the specific ATP generation is decreased (Davis and Johnson 1999). Since MeHg disrupt the mitochondrial production of ATP, the consequent decrease in ATP may augment the apoptotic process, or even convert the death process into necrosis (Elmore 2007). Direct inhibition of GAPDH by MeHg binding directly to the cysteines in this protein have been shown (Eriksson and Svenson 1978), which may explain the molecular rationale behind MeHg-induced effects on this metabolic marker.

Although a clear effect of MeHg on metabolic markers was observed in this study, there were no clear indications that the marine n-3 fatty acids influenced MeHg-induced effects on cell metabolism.

4.3.1 Uptake of MeHg

To confirm that dietary MeHg is indeed taken up into the kidney, Hg was measured directly in kidney tissue and in cells studied *in vitro*. High concentrations of MeHg in kidney, red blood cells, and liver, in Atlantic salmon were observed (Paper I). The fact that the kidney in fish contains a large portion of blood, suggests that there is an uncertainty if the measurement of Hg is indeed of the true kidney content, or of the blood bound MeHg, present in the kidney at the time of measurement. However, the MeHg-uptake studies in ASK and HEK293 cells revealed that kidney derived cells are in fact taking up, and are able to accumulate MeHg (Paper II).

The first explanation of how MeHg was taken up into cells was based on diffusion of methylmercuric chloride (MeHgCl) through a synthetically constructed lipid bilayer (Lakowicz and Anderson 1980). Present-day literature also ascertains that MeHg-Cl uptake is mainly through diffusion because it shows a temperature-independent

uptake (Wu 1996; Heggland et al. 2009). However, uptake through passive diffusion as an *in vivo*-relevant uptake mechanism has been disputed in more recent literature (Simmons-Willis et al. 2002). This is because nearly all MeHg *in vivo* is bound to thiol-containing amino acids or proteins, making it a polar compound with low lipid-solubility not likely to freely cross the lipid membrane of cells. Several studies have therefore used a MeHg-cysteine compound where MeHg is mixed with a slight excess of cysteine, before adding it to cells or experimental animals to mimic *in vivo* conditions (Heggland et al. 2009; Jayashankar et al. 2011). In this study MeHgCl was used because MeHg *in vivo* is not only bound to free cysteine, it can also bind to several other cysteine containing proteins in serum, for example albumin (Yasutake et al. 1990). This means that adding only MeHg-cysteine to cells or animals may not be representative for MeHg *in vivo* conditions. Cell media is an artificial cellular environment containing both cysteine and several serum-proteins. By mixing MeHg-Cl in media before adding it to the cells, it was assumed that most of the MeHg would be bound to free cysteine or other serum proteins, when the cells finally were exposed to the media.

The marine n-3 fatty acids and the n-6 fatty acid ARAs effects on uptake of MeHg in cells were assessed (Paper II). DHA decreased the uptake of MeHg in HEK293 cells, which is consistent with earlier studies performed in different neuronal cells (Kaur et al. 2007; 2008). EPA and ARA did not affect the uptake of MeHg (Paper II). Uptake mechanisms for MeHg have been shown to differ between cell lines (Heggland et al. 2009). Still, the main routes of MeHg-uptake *in vitro* suggested in the present literature is through molecular mimicry via different amino acid carriers such as the methionine carriers large neutral amino acid transporter (LAT) 1 and 2 (Simmons-Willis et al. 2002), the Na^+ dependent amino acid carrier system $B^{0,+}$ (Bridges and Zalups 2006) and the Organic Anion Transporter (OAT) (Zalups and Ahmad 2005) (Figure 5). However, blocking these mechanisms does not abolish the uptake of MeHg, albeit reducing it. DHA is incorporated into the membrane of cells altering its organization, microdomain formation, ion permeability and elasticity (Gorjao et al. 2009). DHA has also been shown to alter membrane protein function through its

incorporation and change of the membrane, however, not necessarily through direct binding (Bruno et al. 2007). The alteration of membrane ion-conductivity and alterations of membrane protein function, such as the receptors responsible for the uptake of MeHg, may be the reason why DHA decreases MeHg uptake in HEK293 cells (Figure 5).

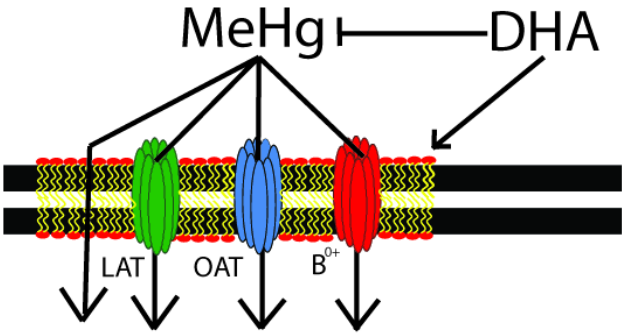

Figure 5 DHA decreased uptake of MeHg in HEK293 cells (Paper II). MeHg is taken up through molecular mimicry via different amino acid transporters for example: methionine carrier large neutral amino acid transporter (LAT) 1 and 2, Organic Anion Transporter (OAT), or Na^+ dependent amino acid carrier system $B^{0,+}$ (B^{0+}) and through diffusion. DHA may decrease uptake through altering these receptors, or generally altering the cell membrane and its permeability.

4.3.2 EPA reduce, while DHA increase MeHg-induced apoptosis

MeHg is strongly cytotoxic and can cause both necrosis and apoptosis in cells (Ceccatelli et al. 2010), but whether the cell death is apoptosis or necrosis can be dependent on dosage and type of apoptotic stimuli (Elmore 2007). To investigate whether marine fatty acids can affect cytotoxicity of MeHg at low doses, apoptosis was assessed using morphological analysis of the nucleus (Paper II). To compare the effects of MeHg on ASK and HEK cells, cytotoxicity was assessed using the novel xCELLigence impedance assay. The xCELLigence technology is based on measurement of impedance through a gold plated electrode. A small current is emitted by the electrode, and if cells are attached to the electrode the current must pass through cells, and the impedance measurement will thus be altered according to cell-adherence and number of cells attached (Paper II). The impact of the fatty acids on MeHg toxicity was not assessed using xCELLigence due to difficulties adjusting

for the high changes in impedance caused by the marine fatty acids in solution. Although ASK cells seemed to be more sensitive to MeHg toxicity, xCELLigence analyses showed relatively similar effects of MeHg on viability in both ASK and HEK293 cells (Paper II).

Morphological analysis indicated that MeHg-induced cell death in ASK cells is mainly due to apoptosis (Paper II). This is consistent with the results from several *in vitro* studies which have reported apoptotic cell death due to MeHg exposure, particularly in the neuronal system, as reviewed by Ceccatelli et al. (2010). In this study, apoptosis after MeHg exposure was concentration dependent in ASK cells (Paper II). Cell death in response to MeHg occurs either through necrosis or apoptosis, where the latter is a controlled cell death, carefully orchestrated through multiple and differing signaling cascades (Elmore 2007). When ASK cells were pre-incubated with DHA an increase of MeHg-induced apoptosis was observed, while EPA pre-incubation decreased MeHg-induced apoptosis. The n-6 fatty acid ARA did not affect MeHg-induced apoptosis (Paper II).

MeHg can induce apoptosis through different molecular pathways, but particularly the classic mitochondrial (intrinsic) mediated apoptosis (Figure 6) is commonly observed (Ceccatelli et al. 2010). A crucial protein family governing intrinsic apoptosis is the B-cell lymphoma 2 (BCL-2) family. In this study, a clear transcriptional up-regulation of the apoptotic regulator *bclx* in response to MeHg was observed (Paper II). ASK cells pre-incubated with DHA also showed a trend toward further increasing the expression of this marker. Although it is not clear whether this particular Atlantic salmon *bclx*-gene is pro- or anti-apoptotic, it reveals the importance of BCL-2 signaling in MeHg induced apoptosis. The BCL-2 family regulates apoptosis through control of mitochondrial permeability and secretion of cytochrome c. The anti-apoptotic BCL-2 proteins BCL-2 and BCLXL resides in the mitochondrial wall inhibiting cytochrome c release, while the pro-apoptotic BAD, BAX, BID and BIM relocates to the mitochondria in response to death signaling and effectuate cytochrome c release (Brunelle and Letai 2009). Release of cytochrome c

activates different proteases named caspases which then cleave key cellular proteins, leading to the classical morphological characteristics of apoptosis (ibid.).

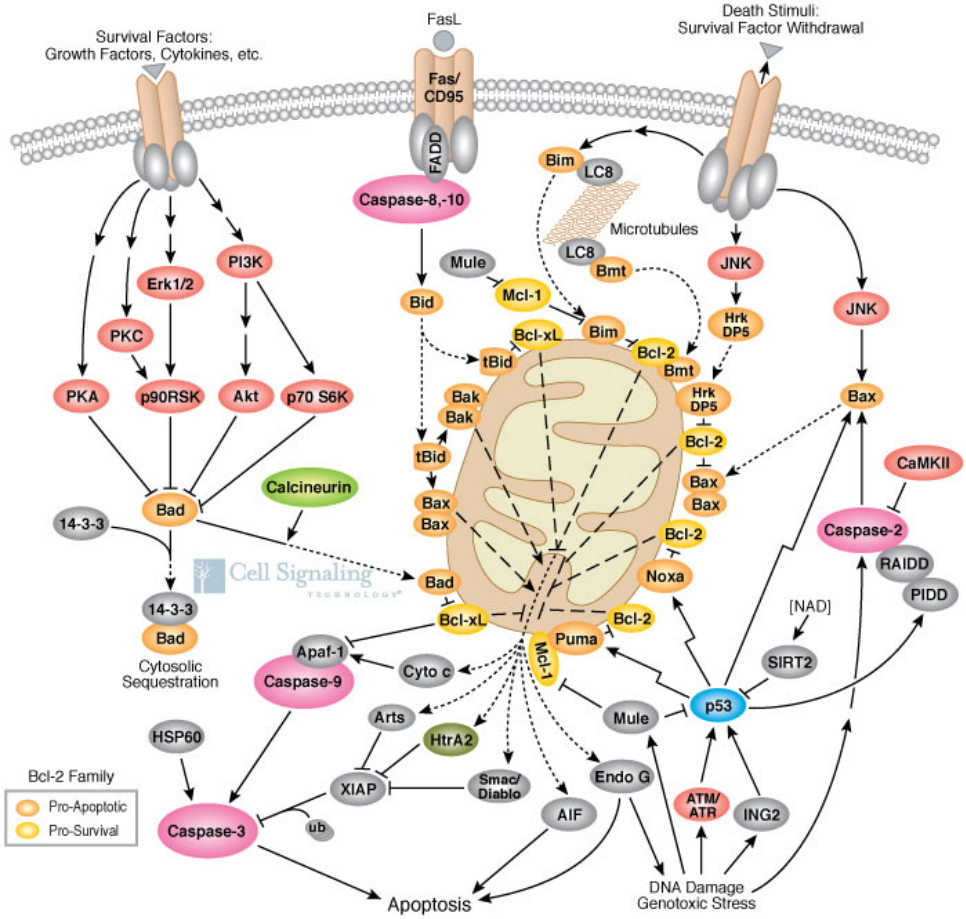

Figure 6. Intrinsic apoptosis, illustrating the role of BCL-2 family proteins. Figure from www.cellsignal.com.

4.3.3 Oxidative stress, the cause for DHA augmented MeHg-induced apoptosis?

Induction of oxidative stress is regarded as one of the most important molecular toxic effects of MeHg (Ceccatelli et al. 2010). Oxidative stress can induce both death-receptor mediated- and intrinsic apoptosis (Figure 6), through several molecular pathways (Circu and Aw 2010; Avery 2011). MeHg can induce oxidative stress through interactions with the glutathione red-ox system and through disruptional

effects on mitochondrial coupled respiration (Yee and Choi 1996; Farina et al. 2011) MeHg induced ROS have also been reported to affect the lysosomal membrane leading to excretion of cathepsins which may in turn induce apoptosis (Ceccatelli et al. 2010).

MeHg can affect state three mitochondrial respiration, which is the stage where oxidative phosphorylation occurs and ATP is produced from ADP (Bourdineaud et al. 2009). An induction (albeit minor) of ATP synthase H^+ transporting mitochondrial F1 complex β (Atp5b) was shown in the present study (Paper I). This protein is involved in the production of ATP from ADP under mitochondrial metabolism, suggesting an effect of MeHg on oxidative phosphorylation in the Atlantic salmon kidney. Yee and Choi (1996) have asserted that MeHg can induce oxidative stress through modulation of complex 3 in the electron transport chain, suggesting that production of ROS may also occur before oxidative phosphorylation. Uncoupling of the electron transport chain can lead to production of ROS and hence oxidative stress (ibid.).

HEK293 cells stably expressing roGFP (Figure 7) were exposed to MeHg and revealed a rapid decline in the calculated red-ox ratio, which can be translated into increased oxidative stress in the cells (Paper II). However, the red-ox ratio quickly recovered, suggesting an intervention of the antioxidant defense system in the cells. By using roGFP real time monitoring, the red-ox balance in cells could be monitored. However, it can be difficult to distinguish between a direct binding of MeHg to roGFP or a general induction of oxidative stress. Still, DHA, which does not have the thiol affinity shown by MeHg, also induced oxidative stress, suggesting that the assay is functional. Whether MeHg binds directly to roGFP or induce oxidative stress, roGFP gives an indication of oxidative inducing effect. To further elucidate oxidative stress in cells in response to MeHg, relevant transcriptional markers were also included in the study (Paper II). Although, no clear indications of oxidative stress could be extrapolated from these transcriptional markers, a differential abundance of the antioxidant protein-markers glutathione peroxidase 2 (Gpx2), thioredoxin (Txn), and hemopexin like protein (Hpx), further confirmed that an oxidative effect of MeHg was observed both *in vivo* and *in vitro* (Paper I and III). MeHg is an

electrophile and can therefore act directly as an oxidative agent (Farina et al. 2011), which may explain the immediate oxidative effects observed (Paper II).

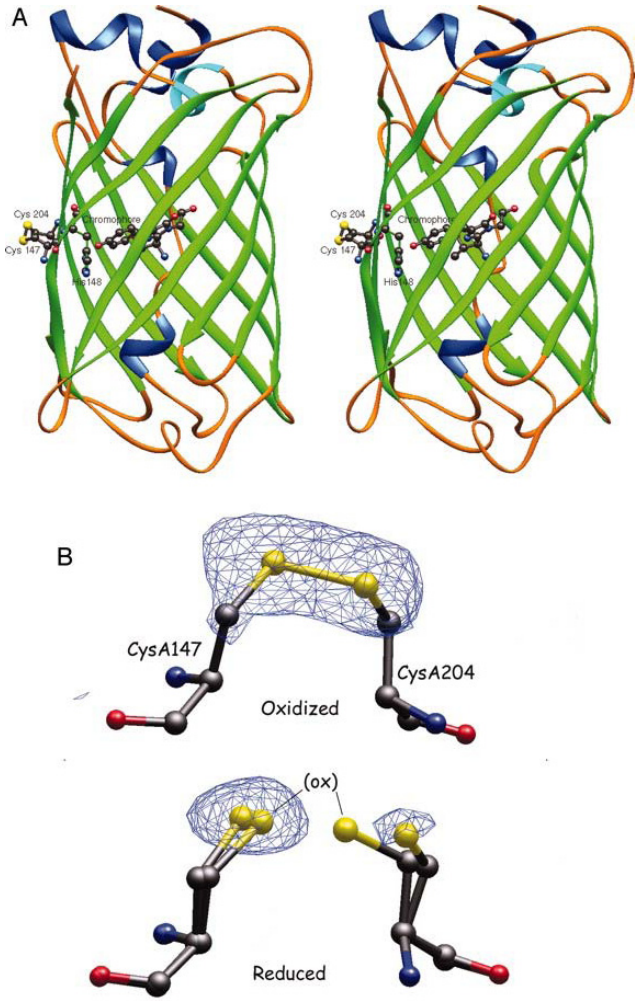

Figure 7. Oxidative stress were assessed using the red-ox Green Fluorescent Protein (roGFP) in HEK293 cells (Paper II). (A) roGFP is a modified version of the normal green fluorescent protein, where serine and glutamine at position 147 and 204 has been replaced by two cysteines to create the roGFP2 C48S/S147C/Q204C (Hanson et al. 2004). (B) The thiol group in these cysteinesreacts to changes in the red-ox environment of the cell, altering the conformation of the roGFP-protein, and subsequently its fluorescent properties. By measuring the emission of the roGFP at two different excitation wavelengths (400nm and 488nm), a ratiometric value representing the red-ox status in the cell can be obtained. Image modified from Hanson et al. (2004).

DHA has also been shown to affect the red-ox environment in cells both in a pro-oxidative and anti-oxidative manner (Kjaer et al. 2008; Shimazawa et al. 2009; Kang et al. 2010). DHA, like MeHg, rapidly decreased the red-ox ratio in HEK293 cells (Paper II), suggesting a pro-oxidative effect of this marine n-3 fatty acid. The pro-oxidative effect of DHA was more consistent in the cells than the effect of MeHg, possibly due to a constant influx of DHA from the membrane and the extracellular milieu. EPA and ARA did not affect the red-ox environment in the cells, suggesting a specific molecular mechanism linked to DHA degradation, producing ROS. In contrast to EPA and ARA, DHA is too large to go directly through mitochondrial β-oxidation, and must first be broken down through peroxisomal β-oxidation (Braiterman et al. 1999). The breakdown of fatty acids in peroxisomes is uncoupled to ATP production and is therefore dependent on other antioxidant defense mechanisms keeping the red-ox balance in cells (Schrader and Fahimi 2006). This could explain why DHA induce oxidative stress, while EPA and ARA do not.

An increased oxidative stress which extends beyond what the antioxidant system of the cell can handle, will cause apoptosis (Circu and Aw 2010). By keeping a consistent oxidative stress in cells, DHA may have depleted the intracellular environment of antioxidant capacity, leaving the system especially vulnerable to a MeHg insult. This may be a pivotal explanation for why DHA increased the MeHg induced apoptosis in ASK cells (Paper II). None of the fatty acids decreased the oxidative stress induced by MeHg, suggesting that there may be other mechanisms than oxidative stress responsible for the positive effects of marine fatty acids on MeHg toxicity.

4.3.4 Calcium homeostasis, the cause for EPA-ameliorating MeHg induced apoptosis?

Several structural proteins were shown to be affected by MeHg in this study (Paper I, II, and III). As mentioned previously, this may be caused by direct binding of MeHg to structural proteins, or as a consequence of apoptotic onset. Strikingly, many of these proteins can also be affected by Ca^{2+}, and consequently through effects on Ca^{2+} homeostasis (Burridge and Feramisco 1981; Wang et al. 2005; Jagatheesan et al.

2010). MeHg is known to disrupt the Ca^{2+} homeostasis in different cell types eventually causing apoptosis (Ceccatelli et al. 2010). Moreover, apoptosis can be initiated directly in response to the intracellular regulation of Ca^{2+} homeostasis (Pinton et al. 2008). In contrast to MeHg, EPA has been shown to maintain Ca^{2+} homeostasis through interaction with the RYR channels located in endoplasmic reticulum (Swan et al. 2003). An increased abundance of the protein sorcin (Sri) in cells pre-incubated with EPA was observed, while MeHg showed a trend of decreasing its abundance (Paper III). SRI is a protein reported in cardiac muscle to inhibit the release of Ca^{2+} through the RYR by direct interaction (Lokuta et al. 1997). Moreover, SRI has also been shown to co-immune-precipitate with voltage-dependent L-type Ca^{2+} channel proteins (Meyers et al. 1998), suggesting an interaction between SRI and Ca^{2+} homeostasis also from the extracellular milieu. Since EPA and MeHg have diverging effects on Sri, this suggests that control over Ca^{2+} homeostasis is the mechanism responsible for the ameliorating effect of EPA on MeHg induced apoptosis (Figure 8).

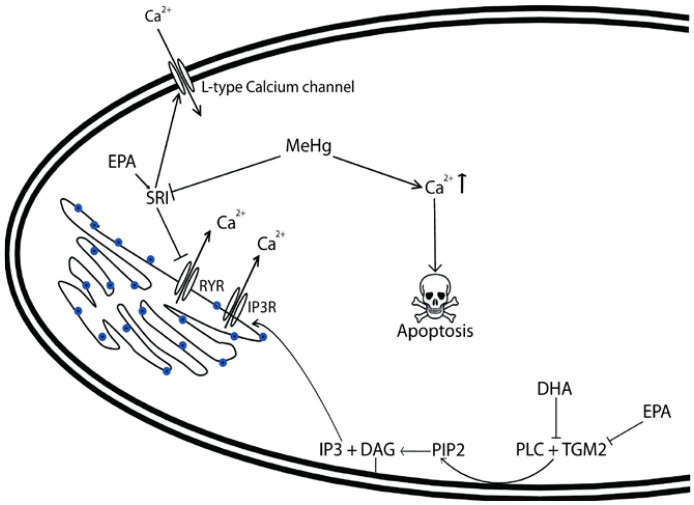

Figure 8. Effects of calcium on MeHg metabolism. EPA increased the expression of sorcin (Sri) a protein known to decrease Ca^{2+} efflux from endoplasmic reticulum. SRI has also been shown to interact with the L-type Ca^{2+} channel, a transporter of Ca^{2+} from the extracellular milieu. Additionally, Phospholipase C (PLC) is also involved in regulation of intracellular Ca^{2+}, and in this study transglutaminase 2 (Tgm2) was differentially regulated in response to DHA and EPA. From the literature, disruption of Ca^{2+} is a known effect of MeHg (Ceccatelli et al. 2010).

Ca^{2+} homeostasis is also controlled through the activity of phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacyl-glycerol (DAG). DAG stays bound to the membrane while IP_3 traverse the cytosol, bind to IP_3-receptor (IP_3R) and induce Ca^{2+} excretion from the endoplasmic reticulum. By inhibiting PLC in MeHg exposed cells, a reduction of toxicity has been observed (Kang et al. 2006), suggesting that its control over Ca^{2+} homeostasis is important in MeHg toxicity. In Paper III we observed a decrease of transglutaminase 2 (Tgm2) in response to EPA and MeHg, while DHA increased its protein abundance. TGM2 can activate PLC (Fesus and Piacentini 2002) and may be another important constituent of the Ca^{2+} homeostasis regulating system (Figure 8).

4.3.5 Cell signalling pathways, concurrent effect of marine n-3 fatty acids and MeHg

Intracellular signal transduction is governed through several pathways where the signal is amplified, quenched or modified in many different manners in the cell (Kholodenko 2006). Effects of both MeHg and the marine n-3 FA on several important signalling molecules related to different signalling pathways were observed (Paper III).

One protein spot recognized as 14-3-3ε (Ywhae) through LC MS/MS was significantly induced by DHA and EPA, while MeHg decreased its abundance (Paper III). At closer investigation of the gel, this protein could be visually estimated at approximately 60 kDa. However, the theoretical mass of 14-3-3ε is approximately 30 kDa. This could be due to dimer formation of the protein (Han et al. 2010), nevertheless, this is not expected under the reductive conditions present during gel preparation. Misidentification of the protein during the LC-MS/MS could also possibly explain the observation. However, the protein has a sufficient score in Mascot, and was recognized through two sequence hits at different places in the protein sequence, which, in addition, is a known 14-3-3 domain. If this protein is indeed the 14-3-3ε, it could be a crucial protein in understanding the effects of marine

fatty acids on MeHg toxicity. Figure 9 (IPA search) illustrates the role of 14-3-3 as an important constituent of several signalling cascades also connected to MeHg toxicity.

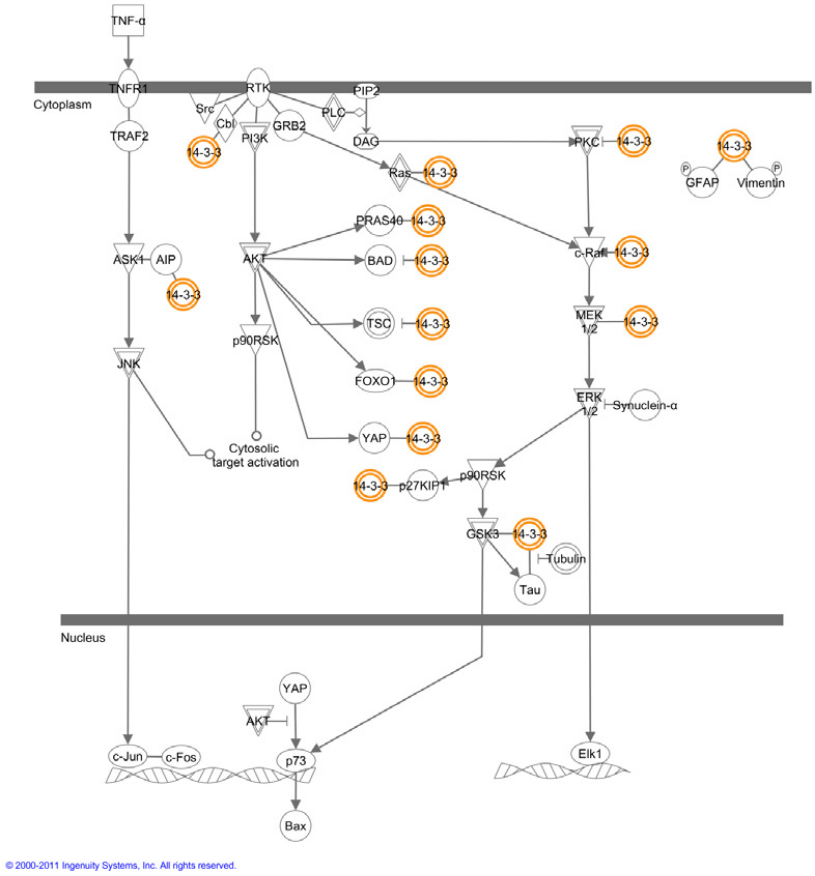

Figure 9. 14-3-3 participates in several signaling pathways. Image obtained from Ingenuity Pathway Analysis (IPA)

A particular important aspect of 14-3-3 is its ability to inhibit BAD induced apoptosis. Since we have shown that BCL-2 linked apoptosis is an often encountered pathway of MeHg-induced apoptosis (Paper II), the ability of 14-3-3 to affect constituents of this pathway, makes it a possible candidate for the modulating effect of marine n-3 fatty acids on MeHg toxicity. Additionally, several of the pathways affected by 14-3-3 are concomitantly affected by MeHg, such as PI3K/Akt (Usuki et al. 2008; Chung et al. 2009) and apoptosis signal-regulating kinase 1/c-jun N-

terminal kinase (ASK1/JNK) signaling pathway (Chen et al. 2006; Zhang et al. 2007), suggesting that an increase in 14-3-3 caused by DHA and EPA may affect MeHg-induced signalling and ultimately its toxicity.

In the experiment described in Paper I, the immunological markers hemopexin like protein (Hpx), alpha-1-antiproteinase-like protein (Serpina7), and galectin 2 (Lgals2) were differentially regulated in response to MeHg, suggesting an effect of MeHg on the Atlantic salmon immune system. The induction of immunological markers by MeHg may suggest a modulated immune system and consequent decreased tolerance for infection, and should be considered when assessing fish health. Also in previous literature, MeHg has been shown to affect the immune system for example by increasing excretion of cytokines, particularly during infection states (Ilbäck et al. 1996; Gardner et al. 2010). Cytokine receptors in cells are responsible for translation of the cytokine-binding into cell signalling, and subsequent cellular response. In addition to its effect on cytokines, MeHg has, as aforementioned, a high affinity for thiols and thereby the amino acid cysteine. Most cytokine receptors have an extracellular domain made up of several cysteines; hence, a direct stimulatory effect of MeHg on these receptors cannot be excluded as a mode of MeHg cell signalling activation. MeHg is known to disrupt the Ca^{2+} homeostasis in cell (Ceccatelli et al. 2010), and in addition to the abovementioned effects of Ca^{2+}, this ion also plays a pivotal role in the effector function of immune cells (Oh-hora and Rao 2008). Hence, the disruptive effect of MeHg on Ca^{2+} homeostasis may also be connected to effects observed on immunological markers in this study (Paper I).

Another aspect of immunological importance in this study is the possible effects on, and of, the eicosanoid system. The eicosanoid pathway has important regulatory effects on several components of the immune system (Kim and Luster 2007). The n-3 fatty acid EPA and the n-6 fatty acid ARA share certain characteristics such as chain length and metabolic breakdown but they differ in one central aspect, namely in their eicosanoid end products. While ARA is the most commonly used substrate for the 2 series eicosanoids, EPA is the substrate for the 3 series eicosanoids. These different series of eicosanoids display very different molecular impacts in cells (Bagga et al.

2003). We observed a decrease in MeHg-induced apoptosis due to EPA pre-incubation, but not with ARA pre-incubation of the ASK cells (Paper II). This may be indicative of the 3 series eicosanoids being the mediator of the anti-apoptotic effect observed (Paper II). Different markers for eicosanoids such as cyclooxygenase 2 (*cox2*), annexin 1 (Anxa1) and coactosin like protein 1 (Cotl1) all showed differential expression in response to MeHg (Paper II and III), suggesting that the eicosanoid system can play an important part in MeHg toxicity, and subsequently also on the modulation shown by EPA. However, a direct link between the eicosanoids and MeHg amelioration were not observed, but should not be excluded as a molecular mechanism for future studies, also in regard to immune responses.

Conclusion

The main finding from this study is:

- The marine n-3 fatty acids DHA and EPA can affect MeHg toxicity *in vitro*.

But in light of the specified aims of this thesis, there are also additional conclusions to be drawn:

- ✓ The toxicity of MeHg in Atlantic salmon kidney is characterized by oxidative stress, effects on metabolism, and effects on immunological markers.
- ✓ Apoptosis plays an important role in cellular MeHg toxicity, particularly the intrinsic apoptotic pathways.
- ✓ The marine n-3 fatty acids DHA and EPA differ in their effects on cellular processes, and consequently on the toxicity of MeHg.
    - Uptake of MeHg in HEK293 cells can be decreased by DHA pre-incubation, but not by EPA.
    - EPA decrease, while DHA increase MeHg-induced apoptosis in ASK cells.
    - DHA increase intracellular oxidative stress and may subsequently augment MeHg toxicity.
    - EPA may ameliorate MeHg toxicity through effects on Ca^{2+} homeostasis.

In conclusion, the importance of understanding nutritional effects in toxicology studies has been underlined by this study. In addition, by including nutrients in traditional toxicology studies, new aspects of molecular mechanisms induced by environmental contaminants can be revealed.

5. Future perspectives

Through the course of this study we have shown that marine n-3 fatty acids can interact with MeHg on a molecular level *in vitro*. Marine n-3 fatty acids affect molecular mechanisms of MeHg-toxicity such as oxidative stress, Ca^{2+} homeostasis, cell signalling and more. Even though this study has explored several aspects of marine n-3 fatty acids impact on MeHg toxicity, it has also generated several interesting aspects to consider for future studies.

This investigation was performed mainly using Atlantic salmon, however, many of the molecular mechanisms observed can easily be transferred also to other species including humans. The modulating effects of marine n-3 fatty acids on MeHg toxicity observed in our study could help explain the great discrepancies observed in epidemiological studies where diet is an underlying effect.

In this study we have also shown that DHA and EPA display different cellular effects, and have different roles in cellular processes. These differences are often not considered when the marine FAs impact on cellular functions is investigated. Dietary sources of DHA and EPA often have varying concentrations and ratio of the two fatty acids, especially in fortified foods and dietary supplements. It is even possible to obtain supplements containing each n-3 fatty acid exclusively. In order to better understand the beneficial effects of the marine n-3 fatty acids, more knowledge about how DHA and EPA differ in physiological functions is of vital importance.

We have investigated marine n-3 fatty acids and MeHg in kidney. However, the molecular mechanisms observed in kidney cells may also be transferable to other organs. The regulation of Ca^{2+} homeostasis for example, could be a crucial ameliorating mechanism of EPA in tissue where Ca^{2+} is crucial for the physiological function of the organ, i. e. Muscle, heart or neurons.

Several proteomic markers and cellular effects have emerged during this study which would be valuable, and also necessary, to investigate in more detail. These are:

- 14-3-3ε, investigate how this protein is involved in both marine n-3 fatty acid and MeHg-induced signalling and eventual apoptosis.
- Sorcin (SRI), evaluate how this protein affects Ca^{2+} homeostasis during MeHg toxicity, also in muscle cells and neuron cells, where Ca^{2+} plays a pivotal role.
- DHAs ability to cause oxidative stress at different concentrations, also *in vivo.*
- Immunological implications of MeHg toxicity.

In conclusion, this study illustrates the importance of incorporating nutrition as a factor in future toxicology studies.

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