THE INTERACTIVE EFFECTS OF N-3 LONG-CHAIN POLYUNSATURATED FATTY

ACIDS AND METHYLMERCURY ON THE CARDIOVASCULAR SYSTEM

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THE INTERACTIVE EFFECTS OF N-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS AND METHYLMERCURY ON THE CARDIOVASCULAR SYSTEM

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DISSERTATION ABSTRACT

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Coldwater marine fish, an excellent source of n-3 long chain polyunsaturated fatty acids (LCPUFA), is often contaminated with the environmental pollutant methylmercury (MeHg). N-3 LCPUFA and MeHg have contrasting effects on the cardiovascular system: n-3 LCPUFA decrease blood pressure and the risk of coronary heart disease, and promote an antiarrhythmic effect; MeHg increases blood pressure, causes irregular heart rate and tachycardia. In a previous study we have shown that animals supplemented with n-3 LCPUFA exhibited significantly lower blood pressure compared to n-3 LCPUFA non-supplemented or MeHg exposed groups. It is possible that the opposite effects of dietary n-3 LCPUFA and MeHg on blood pressure can be explained, in part, through changes in the fatty acid profile of cardiac phospholipids and in oxidative status of heart and liver. Adult Long Evans female rats were exposed from 5 to 18 months of age to 0, 0.5, and 5.0 ppm MeHg in drinking water, and fed either a diet with (+) or without (-) n-3 LCPUFA.

The fatty acid composition of heart phospholipids was analyzed by gas chromatography. The gene expression of the enzymes of pro-oxidant enzyme (NADPH oxidase) and antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase) were determined by RT-PCR. Catalase activity was investigated in liver. Protein oxidation was assessed in heart and liver tissues by measuring protein carbonyl levels. The (+) n-3 LCPUFA diet markedly increased docosahexaenoic acid (DHA) in heart phospholipids as compared to the (-) n-3 LCPUFA diet. Concomitantly, there was a reduction in arachidonic acid (ARA) levels. With a minor exception, there was no effect of mercury exposure on cardiac fatty acid composition. Thus, n-3 LCPUFA significantly improved the fatty acid profiles of the heart, irrespective of mercury concentrations. Dietary n-3 LCPUFA decreased liver and heart gene expression of the pro-oxidant enzyme (NADPH oxidase) ($P \leq 0.001$), whereas MeHg increased the expression of this enzyme ($P = 0.01$) only in the liver. Animals not exposed to MeHg and supplemented with n-3 LCPUFA demonstrated the greatest gene expression of antioxidant enzymes (glutathione peroxidase and catalase). No significant differences were observed in the gene expression of superoxide dismutase in either liver or heart, and in the hepatic catalase activity. In n-3 LCPUFA-fed animals, in the absence of MeHg exposure, cardiac protein oxidation was decreased by 37% compared to the (-) n-3 LCPUFA group. The n-3 LCPUFA incorporation into cardiac phospholipids and their beneficial effect on oxidative status may explain in part the beneficial effects of fish consumption on blood pressure and cardiovascular disease. Therefore, a balance between the risks and benefits of fish intake can be obtained by consumption of fish low in MeHg but rich in n-3 LCPUFA.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I. INTRODUCTION

Cardiovascular disease represents the leading cause of death in the United States. High blood pressure is the single most important risk factor for stroke and the third cause of death among Americans. Approximately one in three adults in the United States has high blood pressure. However, thirty percent of people with high blood pressure do not know they have it. Thus, high blood pressure is being called the "silent killer" (1).

Dietary treatment represents an important factor in the management of high blood pressure. Recommendations of the American Heart Association include the consumption of n-3 polyunsaturated fatty acid (PUFA) from fish and plant sources at least two times a week as a cardiovascular protector (1). Recent evidence reported by the Agency for Healthcare Research and Quality confirms that fish oil consumption can reduce heart attack and other problems related to heart and blood vessels, as well as death from heart disease (2). Five trials used fish oil (EPA+DHA) supplements with a dose ranging between 0.27 and 4.8 g/day. Fish oil significantly reduced all-cause mortality (risk ratio [RR] = 0.79, 95% confidence interval [CI] = 0.66–0.93) and cardiovascular disease outcomes. Most of the twenty-two prospective cohort studies with duration between 4 to 30 years, found that fish consumption reduced all-cause mortality and cardiovascular disease events by 21%.

Although the health benefits of n-3 fatty acids from fish are well known, the risks from environmental toxicants, such as methylmercury, create a challenging paradox for consumers. Cold water marine fish represents the main source of beneficial n-3 long chain polyunsaturated fatty acids (LCPUFA). However, some fish that contain n-3 fatty acids also contain environmental pollutants such as methylmercury (MeHg).

The n-3 LCPUFA exert a beneficial influence on various systems of the human body. As reviewed by Simopoulos (3,4), n-3 PUFA in the adult have beneficial effects on secondary prevention of coronary heart disease, hypertension, type 2 diabetes, ulcerative colitis, Crohn disease, and chronic obstructive pulmonary disease. These beneficial effects are due to antiinflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory properties of n-3 fatty acids. Docosahexaenoic acid (22:6n-3; DHA) is an n-3 LCPUFA that is essential in the diet. The human body is not able to synthesize n-3 unsaturated fatty acids *de novo*; thus, DHA and other n-3 fatty acids are considered dietary essential.

Human exposure to mercury occurs through the intake of fish and sea mammals contaminated with MeHg. After absorption into the bloodstream, MeHg is then transported to other organs (5). Methylmercury is demethylated in tissues and has a very long half life of elimination. Thus, there is the risk of accumulation. Methylmercury accumulates in brain and other organs. Methylmercury adversely affects systems in the adult. Poisoning by MeHg may cause somato-sensory disturbances, poor coordination, vision impairment, and muscle weakness. Methylmercury has negative effects in adults on cardiovascular function. Also, methylmercury may cause developmental, neurological and vision maturation problems in infants. The negative effects of MeHg exposure can

range from subtle neurodevelopmental delays to death depending on the degree of fetal exposure, as reviewed by Castoldi et al. (6). Methylmercury effects were investigated following large outbreaks of toxicity after consumption of contaminated fish in Minamata, Japan (7) and contaminated grains in Iraq (8). Two large prospective epidemiologic studies investigated the neuropsychological effects of prenatal exposure to methylmercury in seafood-eating populations in Faroe Islands (9, 10) and the Seychells (11, 12).

The n-3 polyunsaturated fatty acids (PUFA) and methylmercury have contrasting effects on the cardiovascular system. Holub (13) and Leaf et al. (14) have reviewed the beneficial effects of the n-3 PUFA on the cardiovascular system such as decreasing blood pressure, decreasing the risk of cardiovascular and coronary heart disease and promoting an antiarrythmic effect. Exposure to MeHg increases blood pressure, increases the risk of myocardial infarction, and negatively affects heart rate (15, 16).

The hypothesis of this study is that naturally occurring nutrients found in fish, such as n-3 LCPUFA, will have beneficial effects that counteract the detrimental effects of MeHg on cardiovascular system. The objectives are to assess the interaction between MeHg and n-3 LCPUFA on fatty acid composition of cardiac phospholipids and oxidative status of female rats exposed chronically to n-3 LCPUFA and MeHg.

CHAPTER II. REVIEW OF THE LITERATURE

Polyunsaturated fatty acids: metabolism, general functions and dietary sources

Polyunsaturated fatty acids are long chain fatty acids (most commonly 18-22 carbon atoms) with the first of several double bounds situated at the third or six carbon atom from the methyl end. According to the position of the first double bond, they are separated into two families: n-3 and n-6 fatty acids. Humans are unable to synthesize these long chain unsaturated fatty acids *de novo*; thus, both the n-3 and n-6 families of fatty acids are considered dietary essential. However, humans have the enzymes to convert the 18 carbon atoms fatty acids to 20 or 22 carbon atoms fatty acids but are not able to convert one family of fatty acids to the other. The 18-carbon precursors of the n-3 and n-6 fatty acids families are: alpha-linolenic acid (18:3n-3; ALA) and linoleic acid (18:2n-6; LA), respectively. In the human body, ALA is converted to long-chain polyunsaturated fatty acids (LCPUFA) eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6 n-3; DHA), whereas the major LA-derived fatty acid is arachidonic acid (20:4n-6; ARA). The biochemical pathways involve a series of elongation and desaturation steps and finally peroxisomal β-oxidation (Figure 1).

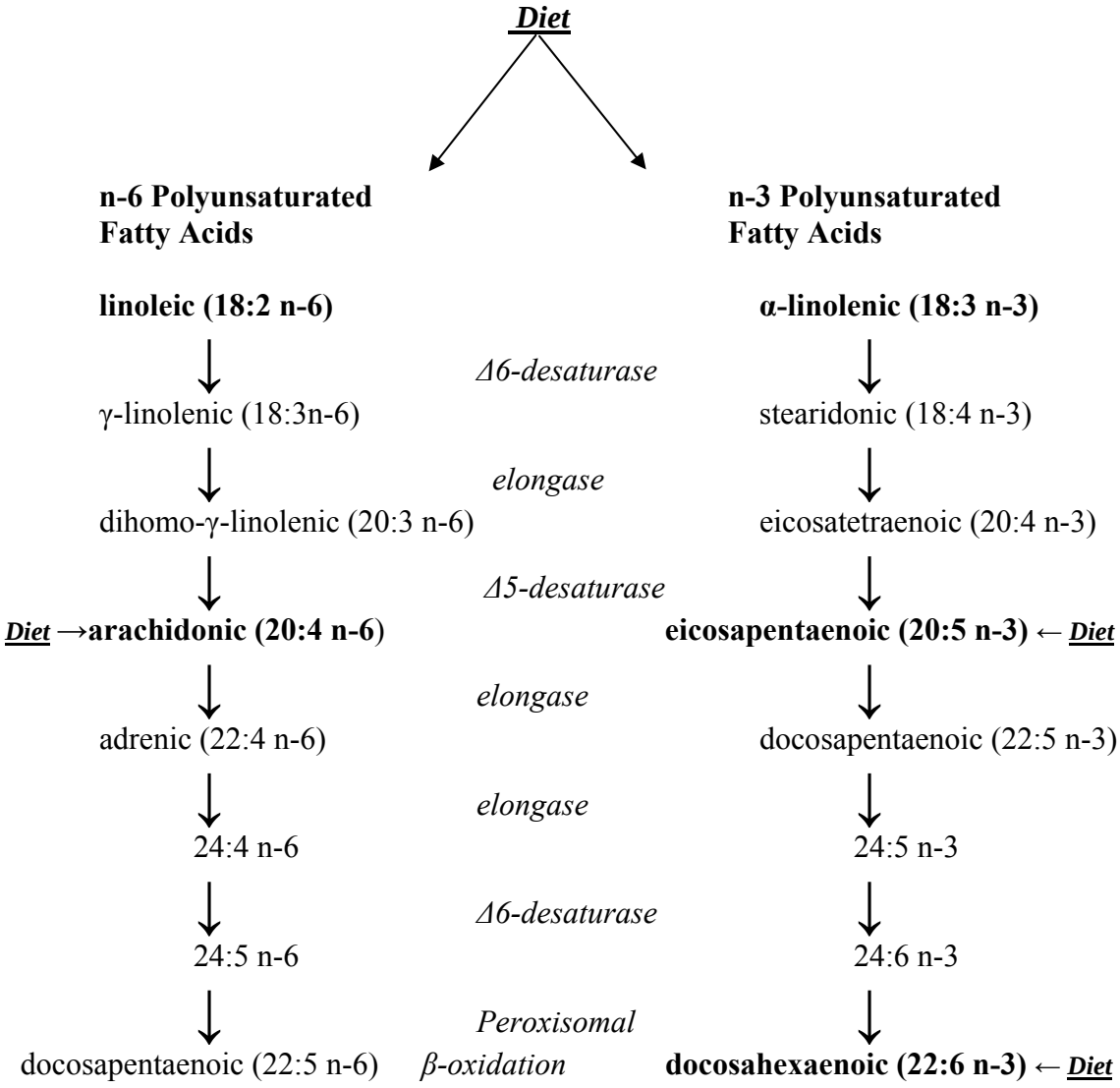

FIGURE 1. Elongation and desaturation of essential fatty acids (adapted from Holub) (13).

Polyunsaturated fatty acids are the main components of cell membranes and contribute to membrane fluidity. These essential fatty acids are necessary for optimal health, growth and development. Innis (17) suggested three mechanisms of action of polyunsaturated fatty acids on metabolic and physiologic pathways: 1) influence of the microenvironment of membrane bilayers, 2) function as signal molecules and precursors for eicosanoids, and 3) effects on gene expression.

The n-3 unsaturated fatty acids, including DHA and EPA, are derived from the diet, being found mainly in fish and other seafood products. Good sources of EPA and DHA are: canned, drained sardines (2.5 g EPA + DHA per 100 g edible portion), Atlantic mackerel and Atlantic salmon (1.8 g and 1.7 g EPA + DHA, respectively, per 100 g edible portions), king mackerel (1.2 g EPA + DHA per 100 g edible portion), Albacore tuna (1 g EPA + DHA per 100 g edible portion) and bluefish (0.8 g EPA + DHA per 100 g edible portion) (18, 3). The n–3 fatty acid recommendation to achieve nutritional adequacy is 0.6–1.2% of energy for ALA; up to 10% of this can be provided by EPA or DHA (19). Flaxseed and flaxseed oil, walnuts and walnut oil, and canola oil are recommended to achieve recommended ALA intakes. Dietary intake of about 500 mg/day of EPA and DHA is recommended for cardiovascular disease risk reduction, and 1 g/day is recommended for the treatment of existing cardiovascular disease.

Health implications of n-3 long-chain polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids of the n-3 family have beneficial effects in the human body while n-3 LCPUFA deficiencies are associated with various disorders. The cardiovascular effects of n-3 fatty acids were best studied and a detailed description of these effects will follow in a subsequent subchapter. The n-3 LCPUFA from fish oil can also help reduce the symptoms of rheumatoid arthritis, according to 12 double-blind placebo controlled studies (20). Osteoporosis could be prevented by the essential fatty acids taken along with calcium (21, 22). A double-blind, placebo-controlled study, conducted on 30 patients with bipolar disorder, sugested that fish oil can produce benefits in bipolar disease, preventing relapse and improving emotional state (23). DHA levels in red blood cells have been correlated with depression in humans (24). High levels of long chain n-3 PUFA lead to an increase in membrane fluidity, the number of insulin receptors and insulin action. Hyperinsulinemia and insulin resistance have been correlated with chronic diseases such as: obesity, type 2 diabetes, hypertension and coronary heart disease (3).

In contrast, a decrease in 20 and 22 carbon PUFA in muscle cell membrane phospholipids is inversely associated with insulin resistance and hyperinsulinemia in patients with coronary heart disease and in normal volunteers (25). Decreases in DHA concentrations in the brain are associated with cognitive decline during aging and with onset of Alzheimer disease (26).

As reviewed by Innis (27), the n-3 polyunsaturated fatty acids are also important for infant growth and development, for visual and neurological development of infants (28), and the inclusion of DHA in the diet improves learning ability (29).

Supplementation with PUFA is especially important for infants who are not breastfed, as breast milk has DHA, and classic formula does not. The need for supplementation of infant formula with DHA, as well as the safety and efficacy of various sources, was studied in a variety of preclinical and clinical trials. Some of the preclinical trials used neonatal pigs as a model. For example, Craig-Schmidt et al. (30) found that dietary supplementation with DHA, AA or both in neonatal pigs caused an accretion of DHA in retinal phospholipids. Fatty acid composition and eicosanoid production in neonatal pigs were significantly affected by the supplementation of infant formula with dietary ARA and DHA (31). Subsequent clinical trials have shown that maternal n-3 fatty acids may have favorable effects on children's physical and mental development (28, 29, 32-35).

Cardiovascular implications of n-3 long-chain polyunsaturated fatty acids

The beneficial effects of n-3 polyunsaturated fatty acids on the cardiovascular system have been determined in numerous human and animal studies. Low risk of cardiovascular and coronary heart disease was observed in fish-eating populations, such as Alaskan Natives, Greenland Eskimos, Inuit of Nunavik and Quebecers, and Japanese people (36-41). High fish intake was reflected in plasma n-3 LCPUFA concentrations.

An inverse association between fish consumption and risk of coronary heart disease and coronary heart disease death has been observed in several epidemiological studies (42-45). For example, in a study conducted in the Netherlands, people over the age of 65 were followed for 17 years, and the relationship between fish consumption and cardiovascular disease was investigated. The people who ate fish (60% of cohort) had a

significantly lower rate of mortality from heart disease compared with elderly who did not eat fish (42).

The Physicians' Health Study was an 11-year follow-up, prospective cohort study of 20,551 United States male physicians free of cardiovascular disease. The incidence of sudden cardiac death (death within 1 h) was assessed. Weekly fish consumption was associated with lower risk of sudden cardiac death (relative risk 0.48; 95% CI 0.24-0.96) compared with consumption of less than 1 fish meal per month (43).

Similarly, the Nurses' Health Study examined the association between fish and long chain n-3 fatty acids and risk of coronary heart disease (CHD) in women (44). The study included 84,688 healthy female nurses who were followed-up for 16 years. A greater consumption of fish and long chain n-3 fatty acids (DHA and EPA) was associated with a lower risk of CHD and CHD death. The relative risks of CHD were: 0.79 for fish consumption 1-3 times per month, 0.71 for once per week, 0.69 for 2-4 times per week, and 0.66 for > 5 times per week (p for trend = 0.001). In addition, the reduction in stroke risk was dose-dependent with a significant reduction in thrombotic and lacunar infarction.

In the Honolulu Heart program study of 8,006 men, low fish intake was defined as consumption of fish less than twice a week and high fish intake as that ≥ 2 times a week (45). There was no difference in the incidence of coronary heart disease between the cohorts, but this lack of association may have been secondary to a threshold effect.

In addition to the epidemiological studies, the impact of n-3 LCPUFA on cardiovascular system has been also evaluated in 4 secondary prevention trials (46-50). The diet and reinfarction trial (DART) randomized 2,033 post–myocardial infarction

Welsh men to one of the following groups: (1) sensible eating (placebo); (2) low fat with an increased polyunsaturated-to-saturated ratio; (3) fatty fish consumption twice a week or 1.5 g fish oil capsules/day; (4) increased dietary fiber; or (5) a combination of the above (46). At 2 years, the group advised to consume fatty fish twice a week or take fish oil capsules had a 29% reduction in all-cause mortality attributable to a reduction in ischemic deaths, whereas nonfatal ischemic heart disease events were not reduced.

Dietary supplementation with 0.9-1.5 g/ day of n-3 PUFA was found to reduce the progression of cardiovascular disease and sudden cardiac death in patients with a history of myocardial infarction (MI). The Gruppo Italiano per lo Studio della Soppravvivenza nell'Infarto miocardico (GISSI) Prevention trial included 11, 324 recently discharged post-myocardial infarction patients randomized to daily supplementation of n-3 fatty acids (EPA/DHA 1:2). After 3.5 years of follow-up, a significant decrease in relative risk for cardiovascular end points (cardiovascular death, non-fatal MI and stroke) was reported (47, 51).

The Indian Experiment of Infarct Survival 4 trial randomized 360 patients with acute coronary syndrome to placebo; fish oil capsules (1.8 g/day EPA + DHA); and 20 g/day of mustard oil (2.9 g/day of α-linolenic acid, ALA) (48). After 1 year, total cardiac events (sudden cardiac death, nonfatal reinfarction, cardiac death) were reduced from 34.7% in the placebo group to 28% in the mustard oil group and to 24.5% in the fish oil group.

The Lyon Heart Study compared a Mediterranean ALA-rich diet with a prudent diet in 608 post–myocardial infarction patients (49). Patients in the experimental group were asked to comply with a specific Mediterranean-type diet. Patients in the control

group received no dietary advice from the researchers but were asked by their physicians to follow a prudent diet. After an average follow-up of 27 months, the Mediterranean ALA-rich diet group achieved a 70% reduction in all deaths, 76% reduction in cardiac death, and a 73% reduction in the primary end point of cardiac death and nonfatal myocardial infarction. A follow-up assessment at 46 months revealed continued good compliance with the Mediterranean ALA-rich diet and similar reductions in total mortality and cardiovascular events (50).

Holub (13) has summarized the beneficial effects and mechanisms of action of EPA and DHA intake on cardiovascular disease. The mechanisms for the cardioprotecive effects of EPA and DHA include: antithrombotic effects by reducing blood platelet reactivity and plasma viscosity, improved endothelial relaxation, inhibitory effect on atherosclerosis and inflammation and lipid lowering effect by reduction in fasting triglyceride and very low density lipoprotein (VLDL) levels and attenuation of postprandial triglyceride response.

The lipid lowering effect of n-3 PUFA has been observed in various studies. Abbey et al. (52) studied the effects of fish oil supplementation on cardiovascular disease prevention in 33 mildly hypercholesterolemic men supplemented with either 14 g/day of linoleic acid as safflower oil, 9 g alpha-linolenic acid as linseed oil, or 3.8 g of n-3 fatty acids as fish oil. Fish oil supplementation decreased plasma triacylglycerols by 24% at six weeks compared to safflower oil supplementation. The fish oil supplemented group had significantly lower concentrations of VLDL, triacylglycerols, thromboxane and total cholesterol. Although, the high-density lipoprotein (HDL) cholesterol decreased slightly in all three groups, the beneficial apo A_I/A_{II} ratio increased by 5%, and the HDL_2/HDL_3

ratio by 28% with the fish-oil diet. Thus, three potentially beneficial changes produced by fish oil supplementation were found: significant decrease in triacylglycerol and VLDL concentration, decrease in thromboxane production, and increase in the HDL_2/HDL_3 ratio.

As reviewed by Kinsella et al. (53), in addition to the lipid lowering effect, n-3 PUFA influence fatty acid membrane profile by replacing arachidonic acid (ARA) with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and thereby affecting eicosanoid metabolism. EPA and DHA inhibit cyclooxygenase and lipoxygenase which leads to a reduction in eicosanoid synthesis such as thromboxane (TXA2) and leukotriene B4 (LTB4). The reduction of proaggregatory and vasoconstrictive TXA2 decreases the thrombotic tendency of platelets. Moreover, the generation of antiaggregatory prostaglandin I3 (PGI3) from EPA is augmented.

The beneficial effect of n-3 PUFA on eicosanoid metabolism may explain the retardation of initiation and progression of atherogenesis by these fatty acids. In addition, the fatty acid profile of the heart may be correlated with the n-3 PUFA antiarrhythmic effect which was studied by Nair et al. (54). The effect of fish oil on n-3 fatty acid composition in normoxic and hypoxic (after occlusion of the left anterior descending artery) myocardium of pigs was investigated. Two groups of female pigs ($n = 7$) were fed for 6 weeks a diet supplemented with either 5 g beef tallow/kg diet (control) or 5 g fish oil/kg diet rich in n-3 fatty acids. The myocardium and myocardial membrane phospholipids of fish oil-fed pigs had increased n-3 PUFA concentration compared with the beef tallow group ($P < 0.001$). The concentration of n-3 unsaturated fatty acids was

greater in the hypoxic compared with normoxic region of the heart, suggesting the possible role of n-3 PUFA in prevention of arrhythmias.

The antiarrhythmic mechanisms of n-3 fatty acids were demonstrated in animal studies and reviewed by Leaf et al. (14). The polyunsaturated fatty acids stabilize the electrical activity of isolated cardiac myocytes by modulating sarcolemmal ion channels. Another mechanism proposed by Leaf et al. (55) is represented by inhibition of voltage-dependent sodium currents and L-type calcium currents. These initiate action potentials in excitable tissues, release of sarcoplasmic calcium stores, which increase cytosolic free calcium and activate the contractile proteins in myocytes.

Human and animal studies have shown that n-3 long chain polyunsaturated fatty acids (LCPUFA) have the ability to lower blood pressure. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the polyunsaturated fatty acids associated with these positive effects on the cardiovascular system. Furthermore, studies have shown that DHA is more powerful than EPA in lowering blood pressure (56, 57). Mori (58) found that DHA, but not EPA, reduces blood pressure and heart rate in mildly hyperlipidemic men who had increased risk of cardiovascular disease. In a double-blind, placebo-controlled trial of parallel design, 59 overweight, mildly hyperlipidemic men were randomized to 4g/day of purified ethyl esters EPA, DHA or olive oil (placebo) capsules for 6 weeks. Purified DHA, but not EPA, significantly reduced ambulatory blood pressure (5.5/3.3 systolic/diastolic mmHg) and heart rate (3.5 ± 0.8 bpm). Prisco and colleagues (59) confirmed that blood pressure significantly decreased after a medium term supplementation (2 months) with this moderate dose of EPA + DHA ethyl esters (4g/day) in mildly hypertensive patients.

The dose-response effect of dietary supplementation with n-3 fatty acids on blood pressure and heart rate was demonstrated in various studies. Two human studies that used various concentrations of n-3 supplementations reported a dose-related effect on lowering the blood pressure and heart rate (60, 61). In a meta-analysis of hypertensive subjects who had a mean fish oil supplementation of 5.6 g/day n-3 fatty acids, there was a significant reduction of 3.4 mmHg in systolic and 1.5 mmHg in diastolic blood pressure (60). There was a dose–response effect of fish oil on blood pressure of -0.66/-0.35 mmHg/g n-3 fatty acids. In the second study, sixty healthy volunteers (25 women and 35 men) were randomly assigned to 3 treatment groups in a double-blind design (61). The subjects received a daily supplement of either 6.6 g n-3 PUFA, 2.0 g n-3 PUFA, or placebo (olive oil) for 12 weeks. Positive associations between DHA concentrations and heart rate variability indexes were found after dietary supplementation with 6.6 g or 2.0 g n-3 PUFA of healthy men in a dose-response relationship.

The dose-dependent antihypertensive effect of DHA has been demonstrated in animal studies also. The DHA antihypertensive effect was investigated in stoke-prone spontaneously hypertensive rats (SHRSP). SHRSP represent a unique animal model. The blood pressure of SHRSP begins to increase at 6-7 weeks of age and at 20 weeks of age, their systolic blood pressure increases to 200 mmHg. SHRSP develop stroke and die around 35 weeks of age, thus having a shorter life span compared with other rat strains. Stroke-prone spontaneously hypertensive rats fed for 14 weeks on 1 % and 5 % DHA supplemented diet demonstrated a decrease in systolic blood pressure to 167.8 and 149.8 mmHg compared with unsupplemented animals (200.9 mmHg) (62). Kimura et al. (63, 64) suggested that long-term administration of DHA attenuated the development of

hypertension in SHRSP through an ameliorative effect of DHA on cholinergic nerve dysfunction. Fourteen weeks of 1% and 5% DHA supplementation of male SHRSP beginning at six weeks of age, significantly reduced blood pressure compared with non-treated animals. The decrease in blood pressure was related to the dose of DHA.

Spontaneously hypertensive rats (SHR) represent another laboratory animal model for study of antihypertensive effect of n-3 PUFA. Bellenger-Germain et al. (65) reported that 11 weeks of dietary supplementation of SHR with a mixture of unsaturated fatty acids suppressed the development of hypertension with the greatest effect in oldest animals. Significantly lower blood pressure and vascular wall thicknesses in the coronary, thoracic and abdominal aorta were observed in SHR fed a DHA enriched diet compared with SHR fed a corn/soybean oils combination diet (66, 67).

Moreover, Chi et al. (68) investigated the effects of dietary n-3 fatty acids on blood pressure and plasma lipids in SHR. SHR were fed for 12 weeks a modified AIN-93G diets containing 1) 8.7% beef tallow + 1.3% corn oil, 2) 9.2% olive oil + 0.8% corn oil, 3) 10% corn oil, 4) 8.5% fish oil + 1.5% corn oil or 5) 3.05% n-3 ethyl ester concentrates + 0.26% docosahexaenoic acid ethyl ester with a mixture of beef tallow, olive oil and corn oil to provide fatty acids similar to diet 4. The increases in blood pressure of SHR fed the diet containing fish oil (diet 4) or n-3 ethyl esters and DHA ethyl ester (diet 5) were significantly attenuated as compared to SHR fed diets containing beef tallow or corn oil. SHR fed diets containing fish oil or n-3 ethyl esters also had significantly reduced plasma triglycerides, cholesterol and HDL-cholesterol/total cholesterol.

De Wilde et al. (69) investigated the effect of long-term dietary n-3 polyunsaturated fatty acids (PUFA) supplementation on blood pressure and brain membrane composition. Hypertensive (SHR) and normotensive (Wistar-Kyoto) rats were provided a placebo (rat chow), or PUFA-enriched diet for 75 weeks. The concentration of cerebral PUFA was lower in the hypertensive than normotensive rats. The authors reported that increased dietary n-3 PUFA supplementation significantly attenuated the development of hypertension in SHR but this effect decreased with age and was not observed in normotensive animals.

Dietary n-3 long-chain polyunsaturated fatty acids and heart fatty acid composition

Long-chain polyunsaturated fatty acids, such as arachidonic acid (20:4 n-6, ARA) and docosahexaenoic acid (22:6 n-3, DHA) are needed for membrane lipid synthesis during growth and development (27). Both fatty acids can be synthesized from dietary essential fatty acids linoleic acid (18:2 n-6, LA) and alpha-linolenic acid (18:3 n-3, ALA), respectively.

The cell membrane consists of a thin lipid bilayer which spontaneously arrange, so that the two layers of phospholipid molecules face the surrounding extracellular and intracellular fluid with their polar heads, and the fatty acyl chains form a continuous hydrophobic interior. The lipid compositions of the two layers of phospholipid are different with a predominance of sphingomyelin and phosphatidylcholine (PC) in the exoplasmic (outer) part and phosphatidylethanolamine (PE) and phosphatidylserine (PS) preferentially located in the cytosolic (inner) part (70, 71). PE and PC constitute approximately 80% of total phospholipid in the rat cardiac tissue (72).

Fatty acids are unevenly distributed among various phospholipid classes. For example, the PE fraction is richer in polyunsaturated fatty acids (PUFA), especially of n-3 series, than PC (72-74, 83-85), whereas the phosphatidylinositol fraction of the membrane selectively incorporates mostly 20:3 and 20:4 fatty acids (86). In addition, the n-3 PUFA are incorporated into cell membranes in a highly selective manner (72, 87). Moreover, DHA preferentially accumulates in the ethanolamine phospholipids of the inner part of the membrane (71, 88).

A detailed review of the fatty acid composition of the heart is represented in Tables 1-6 according to the lipid class and the units used for expression of fatty acid content. In the ethanolamine fraction of phospholipids (Table 1), the major saturated fatty acid (SFA) is 18:0, while 18:1 n-9 is the most prevalent fatty acid in the monounsaturated fatty acid (MUFA) fraction (72-75). Polyunsaturated fatty acids (PUFA) are the predominant fatty acids found in heart PE with n-6 content greater than n-3 fatty acids. The 18:2 n-6 (LA) is known to have a high accretion into heart tissue as compared to other tissues such as brain (66). The predominant long-chain PUFA (LCPUFA) in PE are represented by ARA (20:4 n-6) and DHA (22:6 n-3).

There are a few similarities and differences between PE and PC fractions of the heart phospholipids (Table 2). For example, the difference in the concentration of saturated fatty acids 18:0 and 16:0 is not as visible as was seen in PE. Similar to PE, 18:1 n-9 is the main MUFA as compared to 18:1 n-7 or 16:1 n-7. Moreover, LA is the main PUFA in PC but in a greater concentration than reported in PE. ARA and DHA represent also the main LCPUFA. However, ARA has a greater and DHA a lower representation in

PC than PE fraction. Similar to PE, n-6 fatty acids are the predominant fatty acids over n-3.

The composition of fatty acids in total heart phospholipids follows a similar pattern to phospholipid composition with predominance of 18:0, LA, ARA, DHA, and n-6 fatty acids (Table 3a-f) (54, 76-81). Incorporation of n-3 long chain PUFA into heart phospholipids has been reported for numerous species (73-75, 81, 89, 90).

The fatty acid profile is different among various lipid classes. The phospholipid fraction of rat heart is rich in unsaturated fatty acids while triacylglycerols are rich in monounsaturated fatty acids (Tables 3 and 4). Nikolaidis and colleagues compared the phospholipid and triacylglycerol fatty acid profile of rat (78). ARA, DHA and 22:5 n-3 are the main fatty acids of phospholipids representing 20-26% of total fatty acids as compared to just 1-3 % in triacylglycerols. Monounsaturated fatty acids 16:1 n-7 and 18:1 n-9 constitute 23-32% of total fatty acids in triacylglycerols, as opposed to 3-5% in phospholipids. The 16:0 is the predominant saturated fatty acid in triacylglycerides, whereas 18:0 is the main saturated fatty acid in phospholipid fraction.

The fatty acid composition of heart triacylglycerides, neutral lipids and total lipid are represented in Tables 4-6. As expected, heart triacylglycerides and neutral lipids have similar fatty acid composition (Tables 4, 5) (78, 80). However, the fatty acid content of total lipids is slightly different from that of triacylglycerides and neutral lipids (Table 6) (66, 82). Heart total lipids have a decreased content of 16:0 and greater content of ARA and DHA than of triacylglycerides and neutral lipids.

Dietary interventions can change the fatty acid composition of the heart. For example, ARA content in PE and PC was 50 % lower in rats fed cod liver oil than in rats

fed butter and was partially replaced by the n-3 fatty acids DHA and eicosapentaenoic acid (20:5 n-3, EPA) (73). N-3 LCPUFA-enriched diets markedly increased levels of DHA and EPA while there was a concomitant reduction in ARA (54, 66, 72, 74). High incorporation of LA in heart lipids was found with dietary corn oil or safflower oil (73, 80, 81). The ARA content was not affected by dietary supplementation with its precursor, LA (corn oil). However, an inhibitory effect on ARA was observed with the addition of n-3 PUFA in the diet (cod liver oil and salmon oil) (73, 74). Dietary olive oil causes increased levels of monounsaturated fatty acid 18:1 n-9, whereas saturated fatty acids constitute the major fatty acids in the animals fed a diet rich in coconut oil (80).

TABLE 1a

Fatty acid compositon of heart phosphatidylethanolamine-review[1]

	Benediktsdottir et al., 1988 (73)			Hock et al., 1987 (72)[2]	
		rat			rat
	butter	corn oil	cod liver oil	corn oil	menhaden oil
Fatty acid	10%	10%	10%	5%	5%
16:0	15.0 ± 0.4	12.8 ± 0.6	13.8 ± 0.7	10.24 ± 0.81	12.93 ± 0.42
16:1 (n-7)				0.47 ± 0.19	0.48 ± 0.13
18:0	30.2 ± 0.5	28.6 ± 1.1	27.5 ± 1.2	28.16 ± 0.09 [a]	30.35 ± 0.27 [b]
18:1 (n-9)	7.2 ± 0.2	8.0 ± 0.1	9.1 ± 0.4 [*]	10.82 ± 0.92	8.25 ± 0.78
18:1 (n-7)					
18:2 (n-6), LA	5.0 ± 0.3	9.4 ± 0.6 [*]	6.3 ± 0.4 [†]	10.13 ± 0.28 [b]	2.58 ± 0.38 [a]
20:3 (n-6)					
20:4 (n-6), ARA	18.5 ± 0.2	18.2 ± 1.1	7.7 ± 0.3 [*†]	26.78 ± 0.57 [b]	10.86 ± 0.40 [a]
20:5 (n-3)			2.1 ± 0.1	0.18 ± 0.01 [a]	6.69 ± 0.31 [b]
22:5 (n-3)	3.3 ± 0.3	1.7 ± 0.1 [*]	2.1 ± 0.1 [*]	0.32 ± 0.06 [a]	2.75 ± 0.29 [b]
22:6 (n-3), DHA	17.5 ± 0.8	13.7 ± 0.9	26.6 ± 1.7 [*†]	5.94 ± 1.22 [a]	20.60 ± 1.52 [b]
Others	3.4 ± 0.7	7.7 ± 0.6 [*]	4.8 ± 1.0		
Saturated					
Monounsaturated					
n-6 fatty acids					
n-3 fatty acids					
n-3/n-6				0.23 ± 0.03 [a]	2.25 ± 0.12 [b]

[1]Data reported as % of total fatty acid ± SE.

[2]Values within a row without a common superscript letter are significantly different at $P < 0.01$.

[*] Significant different compared to the butter-fed group at the same time point.

[†] Significant different between rats fed corn oil or cod liver oil at the same time point.

TABLE 1b

Fatty acid compositon of heart phosphatidylethanolamine-review[1]

	Nalbone et al. 1988 (74)[2]			Tvrzicka et al., 1994(75)
		rat		mice
Fatty acid	Control	15% corn	12.5% salmon	
14:0				3.6
16:0	8.8 ± 1.2	8.1 ± 1.0	9.7 ± 1.8	28.94
16:1 (n-7)	0.1 ± 0.09			5.15
18:0	26.9 ± 0.9	27.9 ± 1.9	28.5 ± 0.9	29.64
18:1 (n-9)	4.7 ± 0.2 [b]	4.3 ± 0.2 [b]	3.4 ± 0.2[a]	18.46
18:1 (n-7)	2.8 ± 0.3 [b]	1.4 ± 0.1 [a]	1.9 ± 0.3 [a]	3.86
18:2 (n-6), LA	5.5 ± 0.8 [b]	6.6 ± 0.6 [b]	3.7 ± 0.5 [a]	4.34
20:2 (n-6)	0.1 ± 0.08 [a]	0.3 ± 0.03 [b]	0.1 ± 0.08 [a]	
20:3 (n-6)	0.2 ± 0.03	0.2 ± 0.03		
20:4 (n-6), ARA	30.3 ± 0.6 [c]	24.9 ± 1.2 [b]	8.5 ± 0.5 [a]	0.89
20:5 (n-3)			3.1 ± 0.2	
22:4 (n-6)	2.2 ± 0.3 [a]	3.9 ± 0.3 [b]		
22:5 (n-6)	5.2 ± 1.3 [b]	9.0 ± 0.7 [c]	0.4 ± 0.2 [a]	
22:5 (n-3)	1.2 ± 0.1 [a]	1.3 ± 0.1 [a]	2.7 ± 0.3 [b]	1.07
22:6 (n-3), DHA	11.6 ± 1.0 [a]	13.3 ± 0.5 [a]	37.7 ± 4.2 [b]	3.06
Saturated	35.7 ± 2.5	36.2 ± 3.0	38.2 ± 2.7	62.17
Monounsaturated	7.6 ± 0.3 [b]	5.7 ± 0.3 [a]	5.3 ± 0.2 [a]	30.49
Polyunsaturated	56.8 ± 1.0	58.1 ± 0.8	56.6 ± 0.7	
n-6 fatty acids	43.3	44.7	12.8	5.55
n-3 fatty acids	12.8	14.6	43.5	4.13
n-3/n-6				1.36

[1]Data reported as mol % ± SE.

[2]Values within a row without a common superscript letter are significantly different at $P < 0.01$.

TABLE 2a

Fatty acid compositon of heart phosphatidylcholine-review[1]

	Benediktsdottir et al., 1988 (73)			Hock et al., 1987 (72)[2]	
		rat		rat	
Fatty acid	butter 10%	corn oil 10%	cod liver oil 10%	corn oil 5%	menhaden oil 5%
16:0	22.3 ± 0.2	19.3 ± 0.7	22.1 ± 1.1	20.36 ± 0.6 [a]	25.26 ± 0.6 [b]
16:1 (n-7)				0.63 ± 0.21	1.7 ± 0.24
18:0	22.7 ± 0.4	29.3 ± 0.9	25.3 ± 0.5 [*†]	24.53 ± 0.32	25.61 ± 1.08
18:1 (n-9)	9.6 ± 0.1	7.3 ± 0.1 [*]	11.3 ± 0.3 [*†]	12.08 ± 0.29	12.75 ± 0.49
18:1 (n-7)					
18:2 (n-6), LA	10.5 ± 0.5	13.6 ± 1.0	16.6 ± 1.1 [*]	13.81 ± 0.48 [b]	4.88 ± 0.47 [a]
20:3 (n-6)					
20:4 (n-6), ARA	22.2 ± 0.7	25.0 ± 1.5	10.6 ± 0.9 [*†]	26.27 ± 0.75 [b]	13.93 ± 0.45 [a]
20:5 (n-3)			2.6 ± 0.3	0.10 ± 0.01 [a]	7.92 ± 0.38 [b]
22:5 (n-3)	1.7 ± 0.2	0.6 ± 0.1 [*]	1.2 ± 0.1 [†]	0.05 ± 0.01 [a]	1.43 ± 0.86 [b]
22:6 (n-3), DHA	2.9 ± 0.2	1.9 ± 0.2	6.5 ± 0.9 [†]	0.74 ± 0.04 [a]	4.85 ± 0.59 [b]
Others	3.1 ± 0.8	2.9 ± 0.9	3.8 ± 0.9		
Saturated					
Monounsaturated					
n-6 fatty acids					
n-3 fatty acids					
n-3/n-6				0.03 ± 0.001 [a]	0.75 ± 0.07 [b]

[1] Data reported as % of total fatty acid ± SE.

[2] Values within a row without a common superscript letter are significantly different at $P < 0.01$.

[*] Significant different compared to the butter-fed group at the same time point.

[†] Significant different between rats fed corn oil or cod liver oil at the same time point.

TABLE 2b

Fatty acid compositon of heart phosphatidylcholine-review[1]

	Nalbone et al. 1988 (74)[2]			Tvrzicka et al., 1994(75)
		rat		mice
Fatty acid	Control	15% corn	12.5% salmon	
14:0				0.44
16:0	15.7 ± 0.6	15.0 ± 2.0	16.6 ± 2.6	35.45
16:1 (n-7)	0.4 ± 0.1		0.5 ± 0.09	2.31
18:0	27.1 ± 1.2	28.9 ± 0.4	28.8 ± 1.9	26.46
18:1 (n-9)	4.2 ± 0.4 [b]	3.2 ± 0.2 [b]	2.7 ± 0.2 [a]	21.98
18:1 (n-7)	5.5 ± 0.5 [b]	2.6 ± 0.2 [a]	3.1 ± 0.3 [a]	7.9
18:2 (n-6), LA	8.5 ± 1.4	8.6 ± 1.0	7.3 ± 0.9	3.08
20:2 (n-6)	0.1 ± 0.01 [a]	0.3 ± 0.04 [b]	0.1 ± 0.01 [a]	
20:3 (n-6)	0.2 ± 0.07	0.2 ± 0.03	0.3 ± 0.02	0.21
20:4 (n-6), ARA	34.5 ± 1.0 [b]	35.8 ± 1.9 [b]	22.7 ± 2.1 [a]	0.82
20:5 (n-3)			3.9 ± 0.3	0.21
22:4 (n-6)	0.6 ± 0.07 [a]	1.4 ± 0.3 [b]		
22:5 (n-6)	0.6 ± 0.1 [a]	1.2 ± 0.3 [b]		
22:5 (n-3)	0.4 ± 0.02 [a]	0.5 ± 0.1 [a]	2.1 ± 0.3 [b]	0.33
22:6 (n-3), DHA	1.7 ± 0.2 [a]	1.9 ± 0.4 [a]	11.6 ± 0.9 [b]	0.81
Saturated	43.8 ± 1.9	44.0 ± 2.5	45.5 ± 4.5	62.35
Monounsaturated	10.1 ± 0.3 [b]	6.0 ± 0.1 [a]	6.6 ± 0.2 [a]	32.18
Polyunsaturated	46.1 ± 0.7	50.0 ± 0.8	48.6 ± 0.9	
n-6 fatty acids	44.4	47.3	30	4.11
n-3 fatty acids	2.1	2.1	17.9	
n-3/n-6				1.36

[1]Data reported as mol % ± SE.

[2]Values within a row without a common superscript letter are significantly different at $P < 0.01$.

TABLE 3a

Fatty acid compositon of heart total phospholipids-review[1]

	Rousseau et al., 2003 (76) rat		
Fatty acid	Control	EPA 12.2%	DHA 8.2%
18:2 (n-6) LA	24.7 ± 0.36	23.9 ± 0.49	25.8 ± 0.44
20:2 (n-6)	1.0 ± 0.05	1.2 ± 0.05	1.3 ± 0.10
20:4 (n-6) ARA*	21.1 ± 0.45	15.6 ± 0.40	9.8 ± 0.22
20:5 (n-3)*	0.0 ± 0.01	2.6 ± 0.09	0.7 ± 0.04
22:4 (n-6)			
22:5 (n-3)*	0.6 ± 0.15	4.4 ± 0.12	1.3 ± 0.03
22:6 (n-3) DHA*	5.4 ± 0.43	6.1 ± 0.20	14.3 ± 0.45
Saturated	33.0 ± 0.93	35.0 ± 0.39	36.2 ± 0.40
Monounsaturated	7.2 ± 0.13	6.2 ± 0.14	5.8 ± 0.17
n-6 fatty acids*	48.7 ± 0.56	41.1 ± 0.41	37.4 ± 0.32
n-3 fatty acids*	6.1 ± 0.55	13.2 ± 0.29	16.4 ± 0.47
Polyunsaturated	54.7 ± 0.40	54.3 ± 0.45	53.8 ± 0.41
Other	3.9 ± 0.50	4.5 ± 0.20	4.2 ± 0.20

[1]Data reported as % of total fatty acid ± SE.

* Diet effect ($P < 0.05$).

TABLE 3b

Fatty acid compositon of heart total phospholipids-review[1]

	Pepe et al., 1999 (77) rat					
	Control		n-6 PUFA (11.7%)		n-3 PUFA (11.7%)	
Fatty acid	6 mo	24 mo	6 mo	24 mo	6 mo	24 mo
16:0	9.0 ± 0.29	12.0 ± 0.35	10.0 ± 0.29	14.0 ± 0.69	9.5 ± 0.35	11.0 ± 0.64
18:0	21.3 ± 0.12	24.0 ± 0.87	23.0 ± 1.15	26.0 ± 1.73	10.1 ± 0.72 [bc]	12.0 ± 0.81 [bc]
16:0	0.3 ± 0.07	0.9 ± 0.11	0.22 ± 0.05	0.3 ± 0.12	0.7 ± 0.10	0.8 ± 0.05
18:0	9.4 ± 0.09	10.0 ± 0.39	9.0 ± 0.81	9.0 ± 0.43	9.5 ± 0.40	10.0 ± 0.58
18:2 (n-6)	25.3 ± 0.37	12.0 ± 0.29 [a]	17.0 ± 1.21	11.0 ± 1.44 [a]	12.0 ± 0.58 [bc]	9.0 ± 0.69
20:4 (n-6)	18.0 ± 1.15	29.0 ± 1.44 [a]	24.0 ± 1.44	32.0 ± 1.79 [a]	9.2 ± 0.64 [bc]	12.0 ± 0.58 [bc]
22:4 (n-6)	0.7 ± 0.12	1.1 ± 0.16	1.1 ± 0.18	1.6 ± 0.15	0.1 ± 0.03	0.1 ± 0.01
18:3 (n-3)	0.2 ± 0.01	0.1 ± 0.04	0.2 ± 0.03	0.2 ± 0.03	1.8 ± 0.06	2.0 ± 0.17
20:5 (n-3)	0.3 ± 0.03	0.3 ± 0.01	0.1 ± 0.03	0.1 ± 0.05	6.0 ± 0.17 [bc]	5.2 ± 0.29 [bc]
22:5 (n-3)	2.0 ± 0.16	0.5 ± 0.09 [a]	2.0 ± 0.12	0.8 ± 0.12 [a]	2.2 ± 0.05	5.0 ± 0.58 [bc]
22:6 (n-3)	10.0 ± 0.23	4.0 ± 0.29 [a]	9.0 ± 0.64	2.0 ± 0.43 [a]	27.0 ± 1.01 [bc]	30.0 ± 1.15 [bc]
n-6	44.0 ± 0.84	42.1 ± 1.23	42.1 ± 0.85	44.6 ± 1.58	21.3 ± 0.50 [bc]	21.1 ± 0.33 [bc]
n-3	12.5 ± 0.17	4.9 ± 0.34 [a]	11.3 ± 0.69	3.1 ± 0.44 [a]	37.1 ± 1.1 [bc]	42.2 ± 1.35 [bc]
n-3/n-6	0.29 ± 0.01	0.12 ± 0.01 [a]	0.27 ± 0.02	0.07 ± 0.01 [a]	1.74 ± 0.04 [bc]	2.01 ± 0.09 [bc]

[1]Data reported as mol % ± SE.

[a] Significant different at $P < 0.05$ compared to 6 mo.

[b] Significant different at $P < 0.05$ compared to Control.

[c] Significant different at $P < 0.05$ compared to n-6.

TABLE 3c

Fatty acid compositon of heart total phospholipids-review[1]

	Nikolaidis et al., 2006 (78)	Pepe et al., 1996 (79)[2]		
	rat	rat		
Fatty acid		Control	Saturated 12%	Fish oil 12%
14:0	0.09 ± 0.02	0.1	0.11	0.115
16:0	6.97 ± 0.28	11.23 [b]	9.9 [ab]	9.42 [a]
16:1 (n-7)	0.45 ± 0.15	0.56	0.45	0.79
18:0	18.40 ± 0.84	21.26 [a]	23.29 [b]	24.23 [b]
18:1 (n-9)[3]	5.93 ± 0.46	7.61	7.66	6.63
18:2 (n-6), LA	46.99 ± 1.89	21.63 [c]	13.86 [b]	10.2 [a]
18:3 (n-6)	0.24 ± 0.03	0.12	0.09	0.13
20:2 (n-6)		0.14	0.11	0.09
20:3 (n-6)	0.30 ± 0.04			
20:4 (n-6), ARA	13.63 ± 0.76	17.67 [b]	20.33 [c]	14.13 [a]
20:5 (n-3)	0.09 ± 0.03	0.32 [a]	0.28 [a]	3.21 [b]
22:4 (n-6)		0.36	0.27	<0.1
22:5 (n-3)	1.28 ± 0.38	1.41 [a]	1.84 [b]	1.83 [b]
22:6 (n-3), DHA	5.30 ± 1.46	15.37 [a]	19.46 [b]	27.22 [c]
Saturated		33.88	34.76	34.88
Monounsaturated	6.5 ± 0.8			
n-6 fatty acids*	61.2 ± 1.5	39.8 [c]	34.58 [b]	24.65 [a]
n-3 fatty acids*	6.9 ± 1.7	17.24 [a]	21.39 [b]	32.32 [c]
Polyunsaturated	68.1 ± 0.8	57.04	55.97	56.97

[1] Data reported as mol % ± SE.

[2] Values within a row without a common superscript letter are significantly different at P < 0.05.

[3] 18:1 (n-9 + n-7).

TABLE 3d

Fatty acid compositon of heart total phospholipids-review[1]

	Yaqoob et al., 1995 (80)					
				rat		
Fatty acid	Control	Coconut oil 20%	Olive oil 20%	Safflower oil 20%	Evening Primose 20%	Menhaden oil 20%
16:0	15.9 ± 1.0	10.7 ± 1.0	7.9 ± 0.3	9.1 ± 1.2	12.1 ± 0.9	17.9 ± 1.9
18:0	30.9 ± 1.5	32.6 ± 1.8	22.0 ± 1.5	24.8 ± 2.2	28.5 ± 3.0	23.5 ± 3.2
18:1 (n-9)	9.1 ± 0.8	8.1 ± 0.4	15.2 ± 0.5	6.4 ± 0.8	5.9 ± 0.4	11.9 ± 1.7
18:2 (n-6)	14.5 ± 1.1	11.6 ± 0.6	11.4 ± 0.2	20.8 ± 0.5	19.5 ± 0.5	9.3 ± 2.5
20:4 (n-6)	17.5 ± 1.0	24.6 ± 1.3	29.5 ± 0.6	25.3 ± 1.1	22.3 ± 2.8	1.7 ± 0.4
20:5 (n-3)						18.8 ± 1.6
22:5 (n-3)	1.8 ± 0.3	0.8 ± 0.1	1.7 ± 0.1	3.6 ± 0.3	3.7 ± 0.4	2.6 ± 0.1
22:6 (n-3)	5.5 ± 0.8	7.4 ± 0.6	10.1 ± 0.7	9.6 ± 1.2	9.1 ± 0.8	14.7 ± 2.7

[1] Data reported as mol % ± SE.

TABLE 3e

Fatty acid compositon of heart total phospholipids-review[1]

	Berlin et al., 1998 (81)[2] minipig		
Fatty acid	15% Corn oil	14.25%Corn + 0.75%Menhaden	15% Menhaden
14:0	0.84 ± 0.17 [a]	0.74 ± 0.09 [a]	1.14 ± 0.15 [b]
16:0	31.18 ± 1.21	31.42 ± 1.24	34.67 ± 1.71
16:1 (n-7)	6.54 ± 3.11 [a]	1.88 ± 0.29 [b]	5.49 ± 1.30 [a]
18:0	20.32 ± 2.27	22.52 ± 0.89	22.52 ± 1.24
18:1 (n-9)	20.95 ± 2.37	26.09 ± 1.47	26.42 ± 2.58
18:2 (n-6), LA	12.19 ± 2.13 [b]	13.30 ± 1.00 [b]	7.78 ± 0.92 [a]
18:3 (n-3)	0.65 ± 0.50	0.14 ± 0.03	0.27 ± 0.06
20:0	0.62 ± 0.06	0.62 ± 0.04	0.51 ± 0.09
20:3 (n-6)	1.18 ± 0.86	0.74 ± 0.46	0.37 ± 0.31
20:4 (n-6), ARA	1.33 ± 0.38	0.81 ± 0.16	0.76 ± 0.14
20:5 (n-3)	0.19 ± 0.08	0.15 ± 0.03	0.36 ± 0.10
22:5 (n-3)	0.20 ± 0.19	0.25 ± 0.21	0.10 ± 0.04
22:6 (n-3), DHA	0.04 ± 0.04	0.02 ± 0.01	0.09 ± 0.04
24:0	2.15 ± 1.45	0.55 ± 0.09	0.38 ± 0.08
24:1 (n-9)	0.12 ± 0.05	0.09 ± 0.03	0.16 ± 0.07
Monounsaturated	27.61 ± 4.07	28.06 ± 1.62	32.07 ± 2.37
n-6 fatty acids	14.69 ± 1.98 [b]	14.84 ± 0.91 [b]	8.91 ± 1.08 [a]
n-3 fatty acids	1.08 ± 0.62	0.56 ± 0.25	0.82 ± 0.22
Polyunsaturated	15.77 ± 1.99 [b]	15.41 ± 0.91 [b]	9.73 ± 1.13 [a]

[1] Data reported as mol % ± SE.

[2] Values within a row without a common superscript letter are significantly different at P < 0.05.

TABLE 3f

Fatty acid compositon of heart total phospholipids-review[1]

	Nair et al., 1999 (54) pigs	
Fatty acid	5% Beef tallow	5% Fish oil
16:0	3.0 ± 0.3	3.4 ± 0.2
16:1 (n-7)	0.1 ± 0.0	0.2 ± 0.01
18:0	3.2 ± 0.4	3.5 ± 0.1
18:1 (n-9)	2.6 ± 0.2	2.5 ± 0.2
18:1 (n-7)	1.0 ± 0.1	1.1 ± 0.1
18:2 (n-6), LA	6.1 ± 0.6	3.4 ± 0.2*
18:3 (n-6)	0.1 ± 0.01	0.1 ± 0.01
20:2 (n-6)	0.1 ± 0.01	0.1 ± 0.01
20:3 (n-6)	0.2 ± 0.01	0.1 ± 0.01*
20:4 (n-6) , ARA	3.1 ± 0.2	1.5 ± 0.1*
20:5 (n-3)	0.2 ± 0.03	2.4 ± 0.1*
22:5 (n-3)	0.3 ± 0.03	0.5 ± 0.03*
22:6 (n-3), DHA	0.3 ± 0.02	1.1 ± 0.1*

[1]Data reported as μmol/g heart ± SE. * Diet effect (P < 0.05).

TABLE 4

Fatty acid compositon of heart triacylglycerides-review[1]

Fatty acid	Nikolaidis et al., 2006 (78) rat	Tvrzicka et al., 1994 (75) mice
14:0	1.28 ± 0.50	5.36
16:0	30.09 ± 5.29	37.81
16:1 (n-7)	5.00 ± 2.35	9.44
18:0	6.94 ± 2.04	7.48
18:1 (n-9)	24.40 ± 10.12	33.76
18:1 (n-7)	3.78 ± 0.83	2.71
18:2 (n-6), LA	24.30 ± 8.58	3.42
18:3 (n-6)	0.83 ± 0.52	
20:4 (n-6) ARA	1.35 ± 0.66	
20:5 (n-3)	0.09 ± 0.13	
22:5 (n-3)	0.19 ± 0.08	
22:6 (n-3) DHA	0.23 ± 0.13	
Saturated		50.67
Monounsaturated	33.6 ± 11.5	45.91
n-6 fatty acids	26.6 ± 8.3	
n-3 fatty acids	1.5 ± 0.5	
Polyunsaturated	28.1 ± 8.7	

[1]Data reported as mol % ± SE.

TABLE 5

Fatty acid compositon of heart neutral lipids-review[1]

	Yaqoob et al., 1995 (80) rat					
Fatty acid	Control	Coconut 20%	Olive 20%	Safflower 20%	Evening Primrose 20%	Menhaden 20%
16:0	24.3 ± 0.6	26.2 ± 3.4	19.4 ± 1.6	17.0 ± 1.7	16.7 ± 0.8	27.9 ± 3.2
18:0	13.6 ± 1.7	20.8 ± 1.7	16.5 ± 0.6	16.1 ± 1.1	21.4 ± 1.6	19.9 ± 1.6
18:1 (n-9)	23.5 ± 1.8	19.5 v 2.1	35.2 ± 1.4	17.5 ± 2.4	9.5 ± 0.8	16.2 ± 1.2
18:2 (n-6)	20.4 ± 0.9	13.8 ± 2.6	12.3 ± 0.7	31.6 ± 2.4	33.1 ± 1.6	6.9 ± 0.5
18:3 (n-6)					1.1 ± 0.2	
20:4 (n-6)	8.1 ± 1.1	10.1 ± 1.0	10.2 ± 0.7	14.4 ± 2.4	12.1 ± 1.7	4.4 ± 0.5
20:5 (n-3)						12.4 ± 1.5
22:5 (n-3)	0.7 ± 0.1	1.4 ± 0.1			1.9 ± 0.1	2.9 ± 0.1
22:6 (n-3)	2.8 ± 0.1		2.2 ± 0.2	2.7 ± 0.1	3.3 ± 0.3	14.5 ± 2.1

[1]Data reported as mol % ± SE.

TABLE 6
Fatty acid compositon of heart total lipids-review[1,2]

	Engler et al., 1999 (66)		Alasnier et al., 2002 (82)[3]	
	rat		rat	
Fatty acid	Control	9% DHA	Control 1	Control 2
12:0		0.2 ± 0.0		
14:0	0.9 ± 0.2	1.2 ± 0.1		
16:0	2.3 ± 0.4	2.1 ± 0.2	12.4	12.2
16:1 (n-7)			1.7	1.6
18:0	11.3 ± 0.5	10.6 ± 0.2	18.1	18.4
18:1 (n-9)	6.9 ± 0.9	6.2 ± 0.5	18.4	17.9
18:1 (n-7)	3.4 ± 0.1[b]	2.8 ± 0.0[a]		
18:2 (n-6), LA	29.8 ± 0.8	28.6 ± 0.5	16.9	16.0
18:3 (n-3)	0.6 ± 0.0[b]	0.3 ± 0.0[a]		
20:3 (n-6)	0.2 ± 0.0[a]	0.5 ± 0.0[b]	0.2	0.2
20:4 (n-6) , ARA	22.0 ± 1.0[b]	10.9 ± 0.3[a]	16.7	16.9
20:5 (n-3)		0.6 ± 0.0	0.2	0.2
22:4 (n-6)	1.2 ± 0.0		1.3	1.2
22:5 (n-6)	0.8 ± 0.0			
22:5 (n-3)	2.0 ± 0.1[b]	0.3 ± 0.1[a]		
22:6 (n-3), DHA	5.8 ± 0.3[a]	21.8 ± 0.6[b]	7.9	9.5
Saturated			33.7	33.6
Monounsaturated			20.6	20.1
n-6 fatty acids			35.8	34.9
n-3 fatty acids			8.3	9.9
Polyunsaturated			44.4	45.1

[1] Data reported as % of total fatty acid ± SE.
[2] Values within a row without a common superscript letter are significantly different at P < 0.05.
[3] 18:1 (n-9 + n-7); (22:4 n-6 + 22: 5 n-6); (22: 5 n-3 + 22:6 n-3).

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Health implications of methylmercury

Mercury (Hg) can be released into the air through burning of fossil fuels and can get into both fresh and salt water. Mercury is methylated into methylmercury through the action of micro-organsims (91). Methylmercury (MeHg) represents the organic form of mercury that can accumulate in the food chain of the aquatic system. Long-lived predator fish such as shark, swordfish, king mackerel, and tilefish contain high levels of methylmercury. These larger fish that feed on smaller fish accumulate the highest levels of MeHg and therefore represent the greatest risk to the population (92). The mercury concentrations in the human body are correlated with fish consumption. Fish consumption is greater with higher methylmercury concentrations found in toenails and hair of participants in various cohort studies (93, 94).

Although fish and sea mammals represent the main source of chronic methylmercury exposure of adults, several large outbreaks of acute organic mercury toxicity have been reported. Examples of severe toxicity include consumption of bread mistakenly made from organic mercury-coated grains in Iraq and the industrial release of methylmercury in Minamata Bay in Japan. Poisoning with methylmercury has a long latent period between exposure and the appearance of signs and symptoms. Neurotoxicity and cardiovascular effects were reported after months or even years after exposure to mercury as reviewed by Clarkson and Strain (95).

One of the earliest indicator of neurological damage in adults exposed to methylmercury is represented by the development of paresthesias (96). As reviewed by Clarkson and Strain (95), fine motor changes and sensory function impairment are

observed in subjects exposed to methylmercury. Adults from the Amazon region, exposed to methylmercury from fish, had impaired neuromotor function at hair Hg concentration < 50 ppm. Even when hair Hg concentration was 14 ppm, reduced function on tests of fine motor speed and dexterity and tests of verbal memory were observed (97, 98). Cree Indians living in Canada had poorer performance on a test of rapid, precise movements and poorer eye-hand coordination than control subjects, and the effects were present even with hair Hg concentrations < 50 ppm (99). As reviewed by Mahaffey (100), impairment in sensory function may appear in addition to the fine motor changes. The sensory disturbances occur at lower mercury concentrations compared with the mercury concentrations associated with paresthesias. Thus, sensory impairment can be observed in the absence of paresthesias and can be irreversible.

Methylmercury causes adverse effects on organ development and function of humans and animals during the life span. The developing brain is especially vulnerable to methylmercury exposure. Low doses of methylmercury poisoning during the intrauterine period are associated with neurological and neuropsychological changes in children. An increase in mercury brain: blood ratio was observed in developing rats after a chronic and continuous maternal exposure to 0.5 ppm or 6 ppm MeHg (101).

Infants exposed to methylmercury *in utero* and during infancy exhibited severe mental and physical retardation, cerebral palsy and paralysis (102, 103). Davis et al. (102) presented a case study of methylmercury poisoning from contaminated pork meat in a family that included a pregnant mother. The children had severe neurological dysfunctions. Twenty-two years later, the oldest children developed cortical blindness or constricted visual fields, diminished hand proprioception, choreoathetosis and attention

deficits. The youngest children had quadriplegia, blindness, severe mental retardation and finally died. Harada (104) observed severe neurological problems in children living around Minamata Bay, in Japan. Prenatal exposure to methylmercury was related to microcephaly, cerebral palsy, seizures and mental retardation. Marsh et al. (105) reported similar clinical effects in children exposed *in utero* to methylmercury following the mercury poisoning incident in Iraq. These effects included altered muscle tone, increased deep tendon reflexes, developmental delays and seizures.

Numerous epidemiological studies have been conducted in populations with increased fish and seafood intake. Studies of methylmercury prenatal exposure from seafood consumption conducted in the Faroe Islands and New Zealand have shown adverse neuropsychological effects in school aged children (9, 106, 107). Mercury content in maternal hair, children's hair and cord blood was measured. Prenatal methylmercury poisoning caused widespread neuropsychological dysfunction especially in the areas of language, attention and memory. The neuropathological involvement was less pronounced in visuospatial and motor functions. Mercury content in maternal hair and cord blood at birth was a predictor of neuropsychological dysfunctions in 7 year old children from the Faroe Islands. Grandjean et al. (9) reported a developmental delay of approximately 2 months in highly exposed children. On the other hand, Myers et al. (108) could not find adverse outcomes from fish consumption in 9 year old children from the Republic of Seychelles. The type of seafood consumed with different mercury content can explain the difference in findings from these studies. In the Seychelles, the seafood consumed has lower mercury concentrations (0.3 μg/g) compared with food eaten by

populations in the Faroe Islands or New Zealand (1.6 – 2.2 μg/g). Thus, various concentrations of mercury in the food consumed cause different outcomes.

Methylmercury and the cardiovascular system

Methylmercury exposure has adverse effects on the cardiovascular system, including increased blood pressure, irregular heart rate, tachycardia, and decreased rate variability (5). Accumulation of mercury in the heart produces abnormal cardiac function and blood pressure alterations. Wöβmann et al. (109) reported hypertension and tachycardia in an 11 year old girl after acute mercury intoxication from an unidentified source. The authors suggested that mercury intoxication should be included in the differential diagnosis of tachycardia and hypertension even in cases without clinical signs or history of mercury exposure.

Epidemiological studies conducted in Finland indicate that mercury exposure increased the risk of myocardial infarction (110). The Kuopio Ischemic Heart Disease Risk Factor Study was a large prospective, population-based cohort study. High intake of nonfatty freshwater fish produced an increase in mercury accumulation that was correlated with an increased risk of myocardial infarction, coronary and cardiovascular death in this population. A correlation between mercury concentration and negative health effects was demonstrated in this cohort study of 1,833 Finnish men. The prevalence of acute myocardial infarction and death from coronary heart disease was elevated with increased mercury concentrations in hair and urine (111). In a subsequent study by the same researchers, a correlation between mercury concentrations and accelerated progression of carotid atherosclerosis was observed (112). The risk of acute

myocardial infarction was increased 2.1 fold in the subjects who consumed at least 30 g of fish daily. The hypothetical mechanism proposed by the authors is that lipid peroxidation induced by mercury might increase the risk of cardiovascular and coronary heart disease. On the other hand, the Health Professionals Follow-up Study conducted in 1986-1987 revealed no association between high mercury levels and increased risk of coronary heart disease (94).

Exposure to methylmercury during gestation may have adverse effects on the cardiovascular system later in life. Methylmercury exposure during the prenatal period has been implicated as a cardiovascular risk factor at seven years of age by Sorenson et al. (15) in a cohort study performed in the Faroe Islands. The marine diet of the Faroes includes pilot whale, which increases the methylmercury exposure in this population. With an increase of methylmercury concentration in cord blood from 1 to10 μg/liter, an increase in systolic blood pressure by 14.6 mmHg and an increase in diastolic blood pressure by 13.9 mmHg were observed in seven year old children. The association between mercury exposure and blood pressure was dependent on birth weight. Children with lower birth weight had an increase in systolic and diastolic blood pressure of 20.9/ 24.4 mmHg, and children with birth weight above the median had an increase in systolic and diastolic blood pressure of 9.6/6.7 mmHg. Heart rate variability decreased in boys with increasing mercury exposure. In a follow-up study on this population, at 14 years of age a correlation between methylmercury toxicity and autonomic regulation of heart function was reported (16). Decreased sympathetic and parasympathetic modulation of heart rate variability was associated with methylmercury exposure, and this effect was thought to be caused by the mercury neurotoxicity on the brainstem.

Hypertension and cardiovascular changes have been observed in animals exposed to methylmercury. Systolic hypertension induced by 23-28 days exposure to methylmercury (0.5 mg/kg/day) in Wistar strain rats persisted for more than 1 year (113). Acute toxic effects of mercury were observed after a 2 h continuous exposure of Wistar rats to small concentrations (5 nM) of $HgCl_2$ (114). The mercury exposure had a negative effect on the contractile activity of ventricular myocardium and produced a progressive increase in diastolic blood pressure. The negative effects of methylmercury exposure are thought to be due to cells capacity to concentrate mercury. Tamashiro et al. (115, 116) demonstrated an increase in blood pressure of spontaneous hypertensive rats after exposure to 2 mg/kg per day methylmercury for 26 days.

Interaction between n-3 long-chain polyunsaturated fatty acids and methylmercury on the cardiovascular system

Methylmercury and long chain n-3 polyunsaturated fatty acids (LCPUFA), such as DHA, have similar sources (fish and seafood products) and storage areas in human body. However, they have opposing effects on cardiovascular system.

The opposite effects of MeHg and n-3 LCPUFA on cardiovascular system can be inferred from numerous studies. The Kuopio Ischemic Heart Disease Risk Factor Study, described earlier, showed that high intake of nonfatty freshwater fish produced an increase in mercury accumulation that was correlated with an increased risk of myocardial infarction, coronary and cardiovascular death in this population. (110). However, great intake of n-3 LCPUFA rich fatty-fish was correlated with a beneficial effect on cardiovascular disease risk factors (117). An average daily consumption of 60 g of fatty fish produced higher n-3 fatty acid concentrations and favorable effects on HDL,

triacylglycerols and total:HDL cholesterol in the James Bay Cree population of northern Quebec. As described above, the Nurses Health Study and the Physicians' Health Study showed an inverse association between fish consumption and risk of coronary heart disease and coronary heart disease death (43, 44). The protective effect was stronger for fatal than for nonfatal coronary heart disease.

The interaction of mercury and n-3 fatty acids with respect to cardiovascular disease has not been studied directly, although Guallar et al. (118) in a case-control study of 1,408 men, investigated the association between methylmercury levels in toenail clippings and DHA levels in adipose tissue with the risk of myocardial infarction (MI) among men. The study included 684 men with a diagnosis of MI and 724 men as control group. The average toenail mercury level in controls was 0.25 μg/g and was 15 % greater in the MI men. The risk factor adjusted odds ratio for MI with highest compared to lowest quintile was 2.16. The DHA level was inversely associated with the risk of MI; the odds ratio for the highest vs. the lowest quintile was 0.59. The authors concluded that mercury concentration (as measured in toenails) could be directly associated with the risk of cardiovascular disease, and DHA concentration (as measured in adipose tissue) could be inversely associated with the risk of cardiovascular disease.

Hypotheses

The n-3 LCPUFA and methylmercury have opposing effects on cardiovascular system, thus we proposed a potential interaction between them. In a previous study, we have shown that animals consuming a diet containing n-3 LCPUFA exhibited significantly lower systolic and mean blood pressure compared to control animals in the

absence of MeHg (119). In addition, dietary n-3 LCPUFA prevented the increase in blood pressure with age only in the absence of MeHg exposure.

The opposite effects of dietary fish oil and MeHg on blood pressure could be explained in part, through two mechanisms:

1. Changes in the fatty acid profile of cardiac phospholipids

   Hypotheses: Methylmercury exposure will decrease concentrations of PUFA in the membrane phospholipids by altering enzymatic synthesis of essential fatty acids or their incorporation into phospholipids. Dietary n-3 LCPUFA will increase n-3 fatty acids, decrease n-6 fatty acids content of membrane phospholipid and will counterbalance the methylmercury effects by returning fatty acid concentrations to control levels. Specifically, the objective of this project is to determine if chronic exposure to MeHg alters the heart phospholipid fatty acid profile of the adult female rat, and if dietary n-3 LCPUFA could help overcome the adverse effects of mercury.

2. Changes in the oxidative status

   Hypotheses: Methylmercury exposure will increase oxidative stress and decrease the anti-oxidant defense mechanisms leading to protein oxidative damage. Dietary n-3 LCPUFA will decrease the oxidative stress and increase the anti-oxidant defense mechanisms and counterbalance the negative effects of MeHg.

There are two objectives of this part of study: a) to assess the potential interaction between MeHg and n-3 LCPUFA on pro-oxidant/antioxidant status of rat dams exposed chronically to MeHg and fed a diet with or without n-3 LCPUFA; b) to compare the *in vitro* pro-oxidant effect of MeHg to iron.

CHAPTER III. N-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS AND METHYLMERCURY EFFECTS ON FATTY ACID COMPOSITION OF HEART PHOSPHOLIPIDS

Abstract

Coldwater marine fish, an excellent source of n-3 long chain polyunsaturated fatty acids (LCPUFA), is often contaminated with the environmental pollutant methylmercury (MeHg). N-3 LCPUFA and MeHg have contrasting effects on the cardiovascular system: n-3 LCPUFA decrease blood pressure and the risk of coronary heart disease, and promote an antiarrhythmic effect; MeHg increases blood pressure, causes irregular heart rate and tachycardia. The objective of this study was to analyze the fatty acid composition of the heart in rat dams (n = 8/group) exposed from 5 months to 18 months of age to MeHg (0 ppm, 0.5 ppm, 5 ppm MeHg in drinking water) and fed a diet with (+) or without (-) n-3 LCPUFA. Lipids from the heart were extracted and phospholipids separated into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions using one dimensional thin layer chromatography. The phospholipids fractions were analyzed for fatty acid composition using capillary gas chromatography. Two-way ANOVA and post hoc Tukey's Studentized Range test were used to test for significant differences among experimental groups. The groups receiving n-3 LCPUFA diet exhibited significantly greater concentrations of n-3 PUFA and reduced concentrations of n-6 PUFA in both the

PE and PC fractions compared to the groups receiving no n-3 LCPUFA. The (+) n-3 LCPUFA diet markedly increased the levels of docosahexaenoic acid (DHA) in the heart PE and PC as compared to the (-) n-3 LCPUFA diet. Concomitantly, there was a reduction in arachidonic acid (ARA) levels in PE and PC. With a minor exception, there was no effect of mercury exposure on the fatty acid composition of the heart. Thus, dietary n-3 LCPUFA significantly improved the fatty acid profiles of the heart, irrespective of the mercury concentration.

Introduction

Dietary fish oil is rich in long chain polyunsaturated fatty acids (LCPUFA) of the n-3 family, eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA). Diets rich in n-3 LCPUFA decrease the risk of coronary heart disease and subsequent death (43, 44, 47, 120). Low risk of cardiovascular and coronary heart disease has been observed in fish-eating populations, such as Alaskan Natives, Greenland Eskimos, Inuit of Nunavik and Quebecers, and Japanese people (36-41). Based on the strong evidence that n-3 LCPUFA reduce the risk of cardiovascular disease, the American Heart Association and European Cardiology Society recommend increased intake of fish or fish oil supplementation (121-123). Studies using animal models and isolated cardiomyocytes have shown that n-3 LCPUFA have direct protective effects on the heart (55, 124-128).

Fish, however, is the dominating source of the environmental contaminant, methylmercury, and thus, the risks and benefits to human health of fish consumption have become a controversial issue. Methylmercury (MeHg) represents the organic form

of mercury that can accumulate in the food chain of the aquatic system. Long-lived predator fish such as shark, swordfish, king mackerel, and tilefish contain high levels of methylmercury. These larger fish that feed on smaller fish accumulate the highest levels of MeHg and therefore represent the greatest risk to the population (92).

High mercury levels are associated with increased risk of myocardial infarction and cardiovascular disease (110-112, 129). The prevalence of acute myocardial infarction and death from coronary heart disease are elevated with increased mercury concentrations in hair and urine (111). Moreover, increased levels of mercury accelerate the progression of atherosclerosis in the carotid artery (112).

Methylmercury and n-3 LCPUFA seem to have contrasting effects on the cardiovascular system; however, the interaction of mercury and n-3 fatty acids with respect to cardiovascular disease has not been studied directly. Moreover, the mechanisms by which methylmercury exerts its negative effects are not fully understood. Methylmercury could reduce antioxidant defense mechanisms and promote free radical stress and lipid peroxidation (111) or influence the concentration of fatty acids in the heart. Long-chain PUFA are important constituents of the cell, membranes; thus, the biological properties of membranes and the response of cells to stimuli may depend on the fatty acid composition of membranes.

The working hypothesis of this study is that potentially harmful effects of MeHg on the fatty acid content of the heart could be ameliorated by the protective effects of nutrients, such as n-3 LCPUFA, that are naturally found in fish. Methylmercury exposure will result in alterations of the heart fatty acids by decreasing concentrations of n-3 fatty acids in the membrane phospholipids, and dietary n-3 LCPUFA will counterbalance the

methylmercury effects by returning fatty acid concentrations to control levels. Specifically, the objective of this study was to determine the effect of chronic methylmercury exposure on the fatty acid composition of cardiac phospholipids in rats fed diets with or without n-3 LCPUFA.

Methods

Animals, diets and experimental design

Adult Long Evans female rats were exposed from 5 months to 18 months of age to 0, 0.5, and 5.0 ppm MeHg in drinking water providing approximately 0, 40 and 400 microgram/kg body weight/day of mercury, and fed either a diet without (-) or a diet with (+) n-3 LCPUFA. Thus, the interaction between MeHg and n-3 LCPUFA with respect to membrane phospholipids composition was assessed in female rats exposed chronically to MeHg and n-3 LCPUFA.

The diet for each group (n = 8 animals/group) was based on the AIN-93 formulation but differed in the composition of the dietary fat with fat mixtures replacing the soybean oil (Research Diets, Inc, New Brunswick, NJ). The (-) n-3 LCPUFA diet contained a mixture of palm, safflower, soybean and coconut oils, and the (+) n-3 LCPUFA diet contained the same mixture except that a special EPAX fish oil (Pronova Biocare, Lysaker, Norway) replaced the coconut oil in the mixture. The composition of the diets is given in (Table 7).

Specifically, the fat mixture for the "without (-) n-3 LCPUFA" diet consisted of 42.8% palm oil, 9.2% safflower oil, 15.0% soybean oil and 33% coconut oil, whereas the "with (+)n-3 LCPUFA" diet contained 33% fish oil instead of coconut oil. The mixture of

oils was used in order to keep the levels of linoleic (18:2n-6) and alpha-linolenic (18:3n-3) acid relatively constant (Table 8). The n-6 fatty acid content was almost equal in the two diets as well as the monounsaturated fatty acids. Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) were provided in the (+) n-3 LCPUFA diet at 5% each of total fatty acids, roughly replacing dietary saturated fatty acids. The ratios of total n-6 to n-3 PUFA were 20 for the (-) n-3 LCPUFA diet and 1.8 for the (+) n-3 LCPUFA diet.

Both diets had the same mineral and vitamin content. Thus, the nutrient composition of the two diets differs only in the dietary fat type. Specifically, the diets were similar except that n-3 LCPUFA replaced saturated fatty acids. In order to minimize oxidation, the fish oil was supplemented with an antioxidant mixture of tocopherols Coviox T-70 (10 g/25 kg oil) and Copheral F (14 g/25 kg oil). To maintain tight control over the diets, the same amount of these antioxidants was added to the coconut oil prior to incorporation into the diet. T-butylhydroquinone (TBHQ) was used as per the AIN-93 formulation in both diets. The pelleted diet was kept in a freezer and provided daily.

The animals were exposed to mercury by adding methylmercuric chloride (Alfa Aesar, Ward Hill, MA) to the drinking water. Sodium carbonate (< 5 nmolar) was used to buffer all drinking water. At 18 months of age, the body weight was recorded, the animals were anesthetized, euthanized, and the organs removed. The organs were weighed, blanketed with nitrogen, quick frozen in liquid nitrogen, and stored at -80^0 C for later analysis. All animals were housed in a temperature and humidity- controlled facility with a 12-h light/dark cycle. The Auburn University Animal Care and Use Committee

approved the protocol. The colony was housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility.

Fatty acid analysis of diets and heart phospholipids

Fatty acid composition of diets and heart phospholipids was analyzed in a manner similar to that described by Craig-Schmidt et al. (30) and Huang and Craig-Schmidt (31). Total lipids were extracted from heart and liver tissue, and the diets by a modified Folch et al. (130) procedure using chloroform/methanol (2:1, vol/vol), and BHT (50 mg/L) was added to the extraction medium. Phospholipids were separated from heart lipids by one-dimensional thin-layer chromatography using silica gel plates (Analtech Inc., Newark, DE) and a chloroform: methanol: acetic acid: water (50/25/6/1.5 by volume) solvent system described by Skipsky et. al (131).

The phosphatidylethanolamine (PE) and phosphatidylcholine (PC) fractions were identified using authentic standards (Maytrea Inc., Pleasant Gap, PA), scraped from the thin-layer plate, and transformed into fatty acid methyl esters using a 10% solution of boron triflouride methanol (Supelco, Bellefonte, PA) as described by Morrison and Smith (132).

The fatty acid methyl esters were separated and quantified by a gas chromatographic separation, using a Varian 3900 (Varian Inc., Sugar Land, TX) equipped with: an Omega-wax fused silica capillary column (30m x 0.25 mm i.d., 20 μm film thickness; Supelco, Inc., Bellefonte, PA); a CP8400 autosampler; an 8410 autoinjector; and a flame ionization detector. The injector and flame ionization detector were held at 250^{o}C and 260^{o}C, respectively. Column temperature was programmed with

a continuous gradient (initial temperature of 50^{o}C increasing to 220^{o}C at 4^{o}C/min) followed by a hold time of 35 minutes, and helium (1 mL/min) was used as the carrier gas. Fatty acids were identified by comparison to relative retention times of commercial standards (Matreya, Inc., Pleasant Gap, PA and NuChek Prep, Elysian MN). Fatty acid methyl esters were expressed as percent by weight of total fatty acids by calculating empirical correction factors (133).

Statistics

Two-way ANOVA with post hoc Tukey-Kramer adjustment for multiple comparisons was performed to test for differences in means of fatty acids among all six experimental groups. Results are expressed as means ± SD, and statistical significance was set at $P < 0.05$. Statistical analysis was performed using the SAS 9.1 statistical software (SAS Institute Inc., Cary, NC).

Results

The body weights, heart and liver weights, lipid content of the heart and liver are represented in Table 9. There were no significant differences in animal body weights or weight gain among all groups although a diet effect was observed. No significant differences were observed for the heart weight or total lipid content. There was a mercury effect observed for the liver weight and total lipid content of the liver with greater liver weight and lipid accumulation in animals exposed to mercury.

Effect of n-3 LCPUFA diet and methylmercury exposure on the saturated fatty acid content of phospholipid in the heart

The fatty acid composition, expressed as weight % of total fatty acids (wt% total fatty acids) of heart phosphatidylethanolamine (PE) and phosphatidylcholine (PC), respectively are summarized in Tables 10 and 11. The total content of saturated fatty acids (SFA) was not affected by the (+)n-3 LCPUFA diet or mercury exposure in both PE and PC. Similarly, myristic acid (14:0) and palmitic acid (16:0) content of both PE and PC were not altered by the dietary treatment or methylmercury. However, there was a (+)n-3 LCPUFA main diet effect with decreased levels of lignoceric acid (24:0) in both PE and PC fractions. For example, in the mercury unexposed animals, the 24:0 was less in the PE fraction of animals fed n-3 LCPUFA (0.04 wt% total fatty acids) as compared to 0.3 wt% in the (-)n-3 LCPUFA group. Stearic acid (18:0) content decreased in the PC fraction but not in the PE fraction (from 25.9 wt% total fatty acids in (-)n-3 LCPUFA group to 23.2 wt% in (+)n-3 LCPUFA group).

Effect of n-3 LCPUFA diet and methylmercury exposure on the monounsaturated fatty acid content of phospholipid in the heart

Supplementation of diet with n-3 LCPUFA produced differential effects on monounsaturated fatty acids content in the PE fraction, whereas methylmercury exposure had no effect. Total monounsaturated fatty acid concentration of heart PE was decreased in groups fed (+)n-3 LCPUFA diet as compared to (-)n-3 LCPUFA groups (6.1 wt % compared to 8.6 wt %, respectively). Oleic acid (18:1 n-9) and cis-vaccenic acid (18:1 n-7) concentrations were lower in the (+)n-3 LCPUFA group (3.1 and 2.6 wt% total fatty acids, respectively) compared with (-)n-3 LCPUFA group (4.3 wt% and 3.8 wt% total

fatty acids, respectively). There were no differences between the (-)n-3 and (+)n-3 LCPUFA fed animals in the palmitoleic acid (16:1 n-7) and eicosenoic acid (20:1 n-9) concentrations of heart PE. The monounsaturated fatty acid composition of heart PC was unchanged by the diet or mercury exposure.

Effect of n-3 LCPUFA diet and methylmercury exposure on the polyunsaturated fatty acid content of phospholipid in the heart

Dietary n-3 LCPUFA altered the polyunsaturated fatty acid content of heart PE and PC. Total polyunsaturated fatty acid concentration was increased in groups fed (+)n-3 LCPUFA diet as compared to (-)n-3 LCPUFA group only in heart PE (61.8 wt% versus 59.6 wt% total fatty acids).

For the n-3 series, eicosapentaenoic acid (20:5n-3, EPA), docosapentaenoic acid (22:5 n-3, DPAn-3), and docosahexaenoic acid (22:6 n-3, DHA) concentrations were significantly increased by the dietary (+) n-3 LCPUFA in both PE and PC. For example, EPA concentration increased from trace amounts in (-) n-3 LCPUFA diet animals to 1.9-2.1 wt% of total fatty acids in (+)n-3 LCPUFA groups. Large changes were obtained in DHA concentration. Diet containing n-3 LCPUFA caused an increase in DHA concentration of PE to 35.1 wt% of total fatty acids as compared to (-) n-3 LCPUFA diet for which the value was 13.4 wt % of total fatty acids.

The effects of (+)n-3 LCPUFA diet on the concentrations of n-6 PUFA in heart phospholipids were as follows: linoleic acid (18:2 n-6, LA) content was greater in PE and PC in the (+) n-3 LCPUFA group (7.5 and 13.2 wt% total fatty acids) compared to (-) n-3 LCPUFA group (5.3 and 7.0 wt% total fatty acids). Similarly, 20:3 n-6 (dihomo γ linolenic acid) content was greater in the (+) n-3 LCPUFA group compared to (-) n-3

LCPUFA group in heart PC but not PE. In contrast, arachidonic acid (20:4 n-6, ARA) and other longer chain n-6 fatty acids such as 20:2 n-6, 22:2 n-6, 22:4 n-6 and 22:5 n-6 concentrations were less in the (+) n-3 LCPUFA fed groups as compared to (-) n-3 LCPUFA groups in both PE and PC fractions. For example, the concentration of ARA in PE of (-)n-3 LCPUFA fed animals was 27.9 wt% total fatty acids as compared to 13.2 wt% total fatty acids in (+)n-3 LCPUFA fed animals. In heart PC, ARA concentration decreased from 34.5 wt% total fatty acids in (-) n-3 LCPUFA group to 20.4 wt% total fatty acids in (+) n-3 LCPUFA fed animals.

The methylmercury exposure had no effect on the fatty acid profile in PE and PC with the exception of eicosapentaenoic acid (20:5n-3, EPA) in the PE fraction of the heart (Table 10).

Effect of n-3 LCPUFA diet and methylmercury exposure on the sum of n-3, n-6 fatty acids, and their ratio in heart phospholipids

The sums of n-3 fatty acids, n-6 fatty acids and their ratio were greatly affected in both PE and PC fractions of the heart, by the dietary treatment but not by the methylmercury exposure. Dietary n-3 LCPUFA caused a significant increase in n-3 fatty acids in PE (40.1 wt% total fatty acids) and PC (12.5 wt% total fatty acids) as compared to (-) n-3 LCPUFA diet (15.0 and 2.5 wt% total fatty acids, respectively). The reverse was true for the sum of n-6 fatty acids with a decreased caused by the (+)n-3 LCPUFA diet (21.8 and 34.4 wt% total fatty acids, respectively) as compared to (-)n-3 LCPUFA fed animals (44.5 and 43.9 wt% total fatty acids, respectively).

Comparing the composition of the two phospholipids fractions, sums of n-3 fatty acid was higher in PE as compared to PC of animals fed (-) n-3 LCPUFA diet. The ratio

of n-3/n-6 fatty acids was increased by the (+)n-3 LCPUFA supplemented diet in both PE (1.8) and PC (0.4) as compared to (-)n-3 LCPUFA supplemented diet (0.3 and 0.06, respectively).

Discussion

The objective of this study was to determine the interactive effects of dietary n-3 LCPUFA and MeHg on cardiac phospholipid fatty acid composition. The 2 (dietary treatment) x 3 (methylmercury levels) factorial design allowed the investigation of diet and MeHg main effects, as well as, their possible interaction.

Animals exposed chronically to methylmercury (40 or 400 microgram/kg body weight/day for 1 year) had greater liver weight and hepatic lipid accumulation than unexposed animals. Methylmercury caused fatty livers in adults and infants poisoned in Iraq in 1972 (134). On the other hand, liver disease prevalence in a population from Minamata, Japan, was not significantly different from unexposed population (135). Moreover, MeHg caused no changes in the livers of rats exposed to 0.1 mg MeHg/kg/day for 2 years (136). The MeHg – related fatty changes observed in this study could be due to the biotransformation of Hg in liver. MeHg is slowly demethylated in the liver and intestine, via an unknown mechanism, to Hg^{2+} (5). Mercury that is not demethylated is reabsorbed in the intestine undergoing enterohepatic circulation (96).

We hypothesized that MeHg would alter fatty acid content of the membrane by decreasing the n-3 fatty acids, and that the n-3 LCPUFA diet may protect against MeHg-induced alterations. With a minor exception (20:5 n-3 in PE), no effects of MeHg alone and no interactions between n-3 LCPUFA and MeHg were found. However, we found

that the composition of heart phospholipids was altered by the n-3 LCPUFA supplementation.

Although in the present study, MeHg exerted no effect on fatty acid content of heart phospholipid, MeHg exposure has been shown to alter the fatty acid composition of different tissues such as liver, brain and kidney, by inducing lipid peroxidation (137). Mercury is known to bind with free sulfhydryl groups of membrane proteins to form mercapitides, where it can block specific membrane transport mechanisms and alter selective permeability of the membrane Na^+/K^+ pump through inhibition of Na^+/K^+-ATPase (138). Mercury can also interfere with normal membrane function through reduction in the synthesis of membrane lipids of nervous system (139). However, the current study is the first that investigated the effect of mercury on the fatty acid composition of the heart.

The failure to detect a MeHg alteration of fatty acid content in the heart could be due to the mercury content in the organs or to the exposure doses used. The MeHg exposure doses chosen for this experiment result in low to moderate concentrations in various tissues (101, 140). The mercury exposure level in rat should be greater than the primate exposure, because rat blood has greater density of red blood cells to which mercury is known to bind to a greater extent (141, 142). The highest mercury dose used in the study resulted in a chronic exposure of approximately 400 μg/kg/day. After adjustment for differences in the toxicodynamics of MeHg in rat versus primate blood, this dose is about 2.5 orders of magnitude (141, 142) greater than the current reference dose (RfD) for MeHg (0.1 μg/kg/day) established by the United States Environmental Protection Agency (143).

The cell membrane consists of a thin lipid bilayer which spontaneously arranges itself, so that the two layers of phospholipid molecules face the surrounding extracellular and intracellular fluid with their polar heads, and the fatty acyl chains form a continuous hydrophobic interior. The lipid compositions of the two layers of phospholipid are different with a predominance of phosphatidylcholine (PC) in the exoplasmic (outer) part and phosphatidylethanolamine (PE) preferentially located in the cytosolic (inner) part (70, 71).

Without dietary manipulation, the fatty acid content of both PE and PC described in the present study, are similar to the values found in the literature (74, 77). Fatty acids are unevenly distributed among various phospholipid classes. For example, the PE fraction is richer in PUFA, especially of n-3 series, than PC; finding which is being supported by previous studies (72-74, 83-85). Moreover, as found in the present study and previously reported by others (71, 75, 88), DHA preferentially accumulates in the ethanolamine phospholipid part of the membrane as compared to phosphatidylcholine.

The fatty acids composition of heart membrane phospholipids is sensitive to the dietary fats (72, 76, 77, 79, 144, 145). Dietary treatments of the study consisted of complex mixtures of various oils. The diets for the present study were carefully designed to have n-3 to n-6 fatty acids ratios of 0.5 in (+)n-3 LCPUFA diet and 0.05 (-)n-3 LCPUFA diet while holding the n-6 fatty acid concentration relatively constant between the two diets. Fatty acid composition of the two diets differed also in the concentration of saturated fatty acids. In order to keep the n-6 fatty acids and MUFA relatively constant, the n-3 LCPUFA were replaced in the (+) n-3 LCPUFA diet by saturated fatty acids in

the (-) n-3 LCPUFA diet. Thus, the (-) n-3 LCPUFA diet contained no n-3 LCPUFA whereas the (+) n-3 LCPUFA diet supplied 10 % by weight of EPA + DHA.

The greatest differences observed in the current study were in the polyunsaturated fatty acids concentration of heart phospholipids. The (+) n-3 LCPUFA diet markedly increased the levels of DHA in the heart PE and PC respectively as compared to the (-) n-3 LCPUFA diet. Preferential accumulation of cardiac DHA in rats has been previously reported (125). Moreover, greater content of DHA in PE fraction compared to PC fraction of the heart membrane was found after dietary manipulation with n-3 LCPUFA as have been reported by others (73).

The phospsholipid concentrations of EPA were also increased by the (+) n-3 LCPUFA diet. However, dietary DHA and EPA content (5% each of total fatty acids) had not paralleled their content in the heart phospholipids. This finding is in accordance with previous studies of greater incorporation of DHA versus EPA after dietary interventions with fish oil in heart phospholipid of the rats (73, 74, 79, 146). The low incorporation of EPA into cardiac phospholipid compared to higher level in the diet may be explained by the fact that DHA is being preferentially incorporated into heart lipids versus EPA, or that the elongation and desaturation steps of EPA to DHA are more active in the heart. However, the tissue concentrations of EPA and DHA critical to achieving optimum cardiovascular effects are not known.

Concomitantly, in the present study, there was a reduction in arachidonic acid (ARA) levels in PE and PC. Therefore, dietary n-3 LCPUFA increased the incorporation of n-3 PUFA in heart phospholipids at the expense of n-6 PUFA. Previous studies support the findings that n-3 LCPUFA reduce ARA concentrations in tissue

phospholipids (73, 147). Replacement of ARA by DHA has a positive effect by reducing the incidence of left ventricular fibrillation and sudden death (128, 148).

Dietary interventions may induce different availability of substrates which results in competition in the fatty acid incorporation into phospholipids. The preferential incorporation of PUFA involves phospholipase A_1, A_2, acyltransferases, and transacylases with various substrate specificities as reviewed by MacDonald and Sprecher (149). Competitive effects of n-3 and n-6 fatty acids at level of incorporation into phospholipids were summarized previously by Craig-Schmidt and Huang (150). Acyl-CoA:1-acyl-*sn*-glycero-3 phosphocholine acyltransferase is one of these enzymes which is highly specific for unsaturated fatty acids and has also tissue specificity.

EPA and DHA from fish oil can partially replace ARA in membrane phospholipids. Factors other than the diet can affect PUFA levels in human body only to a limited extent because the tissue content of the essential fatty acids is maintained in a narrow range by regulation of their synthesis. The metabolic pathway of essential fatty acids synthesis takes place in the endoplasmic reticulum and consists of alternating elongation and desaturation steps catalyzed by fatty acid elongase, delta 6 desaturase and delta 5 desaturase enzymes (151). *In vitro* studies on rat liver microsomes have confirmed that the n-6 and n-3 substrates compete at several steps in the microsomal essential fatty acid metabolism pathway (151). The rate-limiting step in the production of ARA and DHA is the desaturation of LA and ALA by delta-6 desaturase; thus the availability of these LCPUFA (ARA and DHA) depends upon the rate of desaturation. Dietary fatty acids are known to influence both delta 5 and 6 desaturases (152).

The differences observed in PUFA content in this study suggest alterations to desaturase enzymes activities. In the present study LA was increased, whereas ARA and 22:5 n-6 (DPA n-6) contents were decreased, indicating the inhibition of delta 6 desaturase by the dietary n-3 LCPUFA. Similar findings have been previously reported (153). In addition to delta-6 desaturase inhibition, n-3 LCPUFA have been also found to inhibit delta-5 desaturase which converts 20:3 n-6 to ARA (153).

Therefore, the possible mechanisms for increased DHA and decreased ARA in heart phospholipid include: a) competition for incorporation into phospholipid; b) competitive effects at elongation or desaturation level.

In the present study, n-3 LCPUFA supplementation induced a significant decrease in ARA concentrations and an increased concentration of its precursor, LA, in cardiac phospholipids. Evidence for an inverse relationship between ARA and LA has been previously reported (31, 154, 155).

Moreover, it has been shown that LA (18:2 n-6) and ALA (18:3 n-3) compete for the same delta 6 desaturase in essential fatty acid synthesis (153, 156, 157). Dietary studies on animals have shown that the n-3 fatty acid ALA is a strong suppressor of n-6 fatty acid metabolism, whereas 10 times as much of the n-6 fatty acid LA is required to give an equal suppression of n-3 metabolism (158). In the current experiment, ALA was not detected in the heart membrane phospholipids. ALA is known to be less effective at inducing biological effects, primarily due to its inefficient conversion to EPA and DHA in humans (159, 160).

We observed a marked reduction in the n-6 to n-3 ratio with n-3 PUFA supplementation in both phospholipid fractions which could be explained by the interplay

of major n-3 and n-6 fatty acid content of the heart. DHA was the major n-3 PUFA while ARA was the major n-6 PUFA in the PE and PC fractions of the heart membrane. The DHA content of the heart achieved with the (+)n-3 LCPUFA diet was significantly greater than that achieved with the (-)n-3 LCPUFA diet in both phospholipid fractions, and the reverse was true for ARA. DHA in the membrane has many unique properties. Dietary DHA incorporation into myocardial membrane has been shown to reduce the incidence and severity of ventricular arrhythmias (161). Modification of membrane fatty acid composition may be one of the mechanisms by which n-3 LCPUFA may have beneficial effects on cardiovascular system. High n-3 long chain PUFA content in the heart has physiological importance by altering myocardial cell membrane fluidity and thus, may affect the contractile properties of the myocardium. It is known that the positive effects of DHA on membrane properties include: fluidity, permeability (162-164), and lipid microdomains formation (165, 166).

In conclusion, dietary supplementation with n-3 long chain polyunsaturated fatty acids had profound effects on the cardiac phospholipid fatty acid composition in a beneficial manner irrespective of methylmercury exposure. Thus, consumption of fish rich in n-3 LCPUFA outweighs the detrimental effects of methylmercury with respect to fatty acid content of the heart.

TABLE 7
Nutrient composition of experimental diets

	(-) n-3 LCPUFA diet	(+) n-3 LCPUFA diet
	g/ 100 g diet	
Protein	14.3	14.3
Carbohydrate	73.1	73.1
Fat	4.0	4.0
kcal/gm	3.9	3.9
Ingredient	g	
Casein, 30 Mesh	140	140
L-Cystine	1.8	1.8
L-Methionine	0.8	0.8
Corn Starch	496.012	496.012
Maltodextrin	125	125
Sucrose	100	100
Cellulose	50	50
EPAX Fish Oil[1]	0	13.2
Coconut Oil[2]	13.2	0
Palm oil	17.12	17.12
Safflower Oil	3.68	3.68
Soybean Oil	6	6
t-Butylhydroquinone	0.008	0.008
Mineral mix[3]	35	35
Vitamin mix[4]	10	10
Choline Chloride	1.38	1.38
Total	1000	1000

[1]AIN-93 Diet for Mature Rodents containing a blend of fish, palm, safflower, and soybean oils (Research Diets, Inc, New Brunswick, NJ).
[2]AIN-93 Diet for Mature Rodents containing a blend of coconut, palm, safflower, and soybean oils (Research Diets, Inc, New Brunswick, NJ).
[3] Supplied per kg of mix: calcium (357 g); copper (0.3 g); iodine (10 mg); iron (6.06 g); magnesium (24 g); manganese (0.63 g); potassium (324.6 g); selenium (10.25 mg); zinc (1.65 g).
[4]Supplied per kg of mix: retinyl palmitate (0.8 g); vitamin D (75 mg); vitamin E (15 g); vitamin thiamin (0.6 g); riboflavin (0.6 mg); pyridoxine (0.7 g); B12 (2.5g); calcium pantothenate (1.6 g); nicotinic acid (3 g); biotin (20mg); folic acid (0.2 g).

TABLE 8

Fatty acid composition of experimental diets

Fatty acid	(-) n-3 LCPUFA[1]	(+) n-3 LCPUFA[2]
	% by weight	
10:0	0.8	0.1
12:0	17.9	1.5
14:0	8.1	3.7
16:0	25.6	29.2
18:0	4.1	4.6
16:1 n-7	0.2	2.5
18:1[3]	22.5	25.9
18:2 n-6	20.0	21.2
18:3 n-3	1.0	1.3
20:5 n-3	-	5.0
22:6 n-3	-	5.2
∑ SFA[4]	56.5	39.1
∑ MUFA[4]	22.7	28.4
∑ PUFA[4]	21.0	32.7
∑ n-3	1.0	11.5
∑ n-6	20.0	21.2
∑ n-6/∑ n-3	20.0	1.8
∑ n-3/∑ n-6	0.05	0.5

[1] Mixture of coconut, palm, safflower, and soybean oils.
[2] Mixture of fish, palm, safflower, and soybean oils.
[3] 18:1 n-9 + n-7.
[4] Abbreviations as follows: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long-chain polyunsaturated fatty acids.

TABLE 9

Body weight, organ weight and organ total lipid content

	(-) n-3 LCPUFA			(+) n-3 LCPUFA		
	MeHg			MeHg		
Parameter[1]	0 ppm	0.5 ppm	5 ppm	0 ppm	0.5 ppm	5 ppm
Body wt (g)[2]*	365.6 ± 17	378.2 ± 17	342.0 ± 17	379.7 ± 17	405.7 ± 17	386.1 ± 17
Wt gain (g)[3]*	74.7 ± 14	85.6 ± 14	53.7 ± 14	84.6 ± 14	110.0 ± 14	93.2 ± 14
Heart wt (g)	0.77 ± 0.03	0.79 ± 0.03	0.72 ± 0.03	0.74 ± 0.03	0.78 ± 0.03	0.72 ± 0.03
R heart wt[2]	0.21 ± 0.01	0.21 ± 0.01	0.21 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.19 ± 0.01
Heart lipid[5]	47.7 ± 2.9	50.4 ± 2.9	45.1 ± 2.9	41.8 ± 2.9	49.0 ± 2.9	46.2 ± 2.9
Liver wt (g)†	0.85 ± 0.04	1.01 ± 0.04	1.0 ± 0.04	0.86 ± 0.04	1.03 ± 0.04	1.02 ± 0.04
R liver wt[2]†	0.23 ± 0.01[a]	0.27 ± 0.02[ab]	0.30 ± 0.02[b]	0.23 ± 0.01[a]	0.26 ± 0.01[ab]	0.27 ± 0.01[ab]
Liver lipid[5]†	62.1 ± 4.4[a]	79.6 ± 10.9[ab]	122.8 ± 13.4[b]	53.3 ± 3.7[a]	83.0 ± 9.3[ab]	87.6 ± 11.6 [ab]

[1] Data are expressed as means ± SE. Groups not connected by the same letter are significantly different; no significant interactions between diet and MeHg by two-way ANOVA with post hoc Tukey-Kramer adjustment were observed for any parameter (n = 8/ group). *N-3 LCPUFA effect. †MeHg effect.

[2] Body weights of 18 month old animals.

[3] Weight gain from 5 to 18 months of age.

[4] Relative organ weight = organ weight / body weight.

[5] Expressed as mg lipid/ g organ weight.

TABLE 10
Heart phosphatidylethanolamine fatty acid composition[1]

	(-) n-3 LCPUFA diet			(+) n-3 LCPUFA diet		
Fatty acid	0 ppm MeHg	0.5 ppm MeHg	5 ppm MeHg	0 ppm MeHg	0.5 ppm MeHg	5 ppm MeHg
14:0	0.3 ± 0.1	0.3 ± 0.3	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
16:0	9.2 ± 0.7	9.2 ± 0.7	9.9 ± 0.8	10.3 ± 0.9	10.0 ± 0.8	10.9 ± 0.6
18:0	22.1 ± 0.7	22.2 ± 1.2	21.5 ± 1.1	21.6 ± 1.3	21.9 ± 1.0	21.2 ± 1.2
24:0*	0.3 ± 0.1^{b}	0.3 ± 0.1^{b}	0.4 ± 0.1^{b}	0.04 ± 0.04^{a}	0.1 ± 0.03^{a}	0.1 ± 0.03^{a}
16:1n-7	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
18:1n-9*	4.3 ± 0.6^{b}	4.4 ± 0.4^{b}	4.3 ± 0.4^{b}	3.1 ± 0.3^{a}	3.0 ± 0.3^{a}	3.1 ± 0.3^{a}
18:1n-7*	3.8 ± 0.4^{b}	3.7 ± 0.4^{b}	3.8 ± 0.4^{b}	2.6 ± 0.2^{a}	2.7 ± 0.3^{a}	2.8 ± 0.2^{a}
20:1n-9	0.1 ± 0.04	0.1 ± 0.04	0.1 ± 0.1	0.04 ± 0.04	0.1 ± 0.03	0.1 ± 0.03
18:2n-6*	5.3 ± 1.7^{a}	5.5 ± 1.5^{a}	4.6 ± 1.2^{a}	7.5 ± 1.5^{b}	6.1 ± 0.8^{a}	6.2 ± 1.1^{a}
20:2n-6*	0.2 ± 0.1^{b}	0.2 ± 0.02^{b}	0.2 ± 0.01^{b}	0.02 ± 0.03^{a}	0.1 ± 0.01^{a}	0.02 ± 0.04^{a}
20:3n-6	0.2 ± 0.04	0.2 ± 0.01	0.2 ± 0.04	0.2 ± 0.1	0.2 ± 0.03	0.2 ± 0.03
20:4n-6*	27.9 ± 1.6^{b}	27.1 ± 2.2^{b}	26.0 ± 2.1^{b}	13.2 ± 1.6^{a}	13.2 ± 1.8^{a}	12.7 ± 1.5^{a}
22:2n-6*	0.1 ± 0.06	0.1 ± 0.05	0.1 ± 0.06	Tr	Tr	Tr
22:4n-6*	2.7 ± 0.3^{b}	2.4 ± 0.4^{b}	2.5 ± 0.2^{b}	0.2 ± 0.04^{a}	0.3 ± 0.1^{a}	0.2 ± 0.1^{a}
22:5n-6*	8.2 ± 1.5^{b}	8.6 ± 1.6^{bc}	10.1 ± 1.3^{c}	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}
20:5n-3*†‡	Tr	Tr	Tr	1.9 ± 0.3^{b}	1.5 ± 0.4^{a}	1.5 ± 0.3^{a}
22:5n-3*	1.7 ± 0.5^{a}	1.3 ± 0.5^{a}	1.2 ± 0.2^{a}	3.1 ± 0.3^{b}	3.2 ± 0.3^{b}	2.9 ± 0.4^{b}
22:6n-3*	13.4 ± 2.5^{a}	14.1 ± 2.3^{a}	14.5 ± 2.2^{a}	35.1 ± 1.5^{b}	36.6 ± 2.3^{b}	37.1 ± 2.6^{b}
∑SFA	31.9 ± 0.8	32.0 ± 1.5	32.0 ± 1.8	32.1 ± 1.3	32.2 ± 1.5	32.3 ± 1.7
∑MUFA*	8.6 ± 1.1^{b}	8.5 ± 0.7^{b}	8.6 ± 0.8^{b}	6.1 ± 0.5^{a}	6.1 ± 0.5^{a}	6.3 ± 0.4^{a}
∑PUFA*	59.6 ± 1.5^{a}	59.5 ± 1.7^{a}	59.4 ± 1.3^{a}	61.8 ± 1.4^{b}	61.8 ± 1.5^{b}	61.5 ± 1.9^{b}
∑n-6*	44.5 ± 1.8^{b}	44.1 ± 1.8^{b}	43.6± 2.9^{b}	21.8 ± 1.9^{a}	20.5 ± 2.0^{a}	20.0 ± 1.9^{a}
∑n-3*	15.0 ± 2.8^{a}	15.4 ± 2.3^{a}	15.8 ± 2.0^{a}	40.1 ± 1.5^{b}	41.3 ± 2.4^{b}	41.5 ± 2.9^{b}
∑n-6/n-3*	3.0 ± 0.7^{b}	2.9 ± 0.7^{b}	2.8 ± 0.5^{b}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}
∑n-3/n-6*	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.01^{a}	1.8 ± 0.2^{b}	2.0 ± 0.3^{b}	2.1 ± 0.3^{b}

[1]The data are expressed as wt % of total identified fatty acids. Results are given as mean ± SD. Means within a row with different superscript letters are significantly different. *(+)n-3 LCPUFA diet effect. †MeHg effect and interaction effect between (+)n-3 LCPUFA and MeHg by two-way ANOVA with post hoc Tukey-Kramer adjustment (n = 8/ group). ∑, the sum; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long-chain polyunsaturated fatty acids; Tr, trace amounts.

TABLE 11
Heart phosphatidylcholine fatty acid composition[1]

	(-) n-3 LCPUFA diet			(+) n-3 LCPUFA diet		
Fatty acid	0 ppm MeHg	0.5 ppm MeHg	5 ppm MeHg	0 ppm MeHg	0.5 ppm MeHg	5 ppm MeHg
14:0	0.2 ± 0.1	0.2 ± 0.04	0.2 ± 0.04	0.2 ± 0.04	0.2 ± 0.04	0.2 ± 0.1
16:0	17.1 ± 1.5	17.6 ± 1.0	18.3 ± 1.7	19.0 ± 1.9	19.3 ± 1.6	19.8 ± 1.9
18:0*	25.9 ± 1.4[b]	25.2 ± 1.3[b]	25.6 ± 0.1[b]	23.2 ± 0.9[a]	23.4 ± 0.9[a]	23.4 ± 1.0[a]
24:0*	0.02 ± 0.03	0.06 ± 0.1	0.06 ± 0.04	Tr	Tr	Tr
16:1n-7	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.6 ± 0.06	0.6 ± 0.1
18:1n-9	5.0 ± 0.8	5.2 ± 0.8	5.1 ± 0.5	5.4 ± 0.8	4.7 ± 0.5	4.9 ± 0.8
18:1n-7	4.9 ± 0.7	5.2 ± 0.6	4.9 ± 0.6	4.6 ± 0.6	4.6 ± 0.6	4.3 ± 0.6
20:1n-9	0.1 ± 0.03	0.1 ± 0.01	0.1 ± 0.04	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02
18:2n-6*	7.0 ± 2.5[a]	7.3 ± 3.1[a]	6.0 ± 1.4[a]	13.2 ± 2.7[b]	9.9 ± 2.8[b]	10.2 ± 2.6[b]
20:2n-6*	0.3 ± 0.06[b]	0.2 ± 0.04[b]	0.2 ± 0.07[b]	0.1 ± 0.01[a]	0.1 ± 0.03[a]	0.1 ± 0.02[a]
20:3n-6*	0.3 ± 0.01[a]	0.3 ± 0.04[a]	0.3 ± 0.1[a]	0.5 ± 0.1[b]	0.4 ± 0.1[b]	0.5 ± 0.1[b]
20:4n-6*	34.5 ± 3.3[b]	33.6 ± 3.7[b]	34.3 ± 1.8[b]	20.4 ± 3.0[a]	22.8 ± 3.7[a]	22.6 ± 2.7[a]
22:2n-6*	0.01 ± 0.02	0.02 ± 0.03	0.02 ± 0.02	Tr	Tr	Tr
22:4n-6*	0.8 ± 0.1[b]	0.7 ± 0.1[b]	0.6 ± 0.2[b]	0.1 ± 0.02[a]	0.1 ± 0.1[a]	0.1 ± 0.02[a]
22:5n-6*	1.0 ± 0.2[b]	1.2 ± 0.4[b]	1.3 ± 0.2[b]	0.2 ± 0.03[a]	0.2 ± 0.03[a]	0.2 ± 0.04[a]
20:5n-3*	Tr	Tr	Tr	2.1 ± 0.3	1.8 ± 0.5	1.8 ± 0.4
22:5n-3*	0.6 ± 0.2[a]	0.5 ± 0.1[a]	0.5 ± 0.1[a]	2.0 ± 0.5[b]	2.3 ± 0.3[b]	2.0 ± 0.3[b]
22:6n-3*	1.9 ± 0.4[a]	2.2 ± 0.6[a]	1.9 ± 0.4[a]	8.4 ± 1.7[b]	9.6 ± 1.4[b]	9.3 ± 1.6[b]
∑SFA	43.2 ± 0.6	43.1 ± 0.7	44.2 ± 1.8	42.4 ± 1.4	42.9 ± 0.9	43.4 ± 1.2
∑MUFA	10.4 ± 1.6	10.9 ± 1.4	10.6 ± 1.0	10.7 ± 1.4	10.0 ± 1.0	9.9 ± 1.3
∑PUFA	46.3 ± 1.6	46.0 ± 1.2	45.1 ± 2.0	46.9 ± 2.4	47.1 ± 1.8	46.7 ± 2.2
∑n-6*	43.9 ± 1.2[b]	43.3 ± 1.0[b]	42.8 ± 1.7[b]	34.4 ± 1.6[a]	33.5 ± 2.0[a]	33.6 ± 1.1[a]
∑n-3*	2.5 ± 0.5[a]	2.7 ± 0.6[a]	2.4 ± 0.4[a]	12.5 ± 2.1[b]	13.6 ± 2.0[b]	13.1 ± 2.0[b]
∑n-6/n-3*	17.6 ± 3.7[b]	16.1 ± 4.8[b]	17.8 ± 2.8[b]	2.8 ± 0.5[a]	2.5 ± 0.5[a]	2.6 ± 0.5[a]
∑n-3/n-6*	0.06 ± 0.01[a]	0.06 ± 0.01[a]	0.06 ± 0.01[a]	0.4 ± 0.1[b]	0.4 ± 0.1[b]	0.4 ± 0.1[b]

[1]The data are expressed as wt % of total identified fatty acids. Results are given as mean ± SD. Means within a row with different superscript letters are significantly different. *(+)n-3 LCPUFA diet effect by two-way ANOVA with post hoc Tukey-Kramer adjustment (n = 8/ group). No effects of MeHg or interaction between diet and MeHg were observed. ∑, the sum; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long-chain polyunsaturated fatty acids; Tr, trace amounts.

CHAPTER IV. N-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS AND METHYLMERCURY EFFECTS ON OXIDATIVE ENZYMES OF CARDIAC AND HEPATIC ANIMAL TISSUE

Abstract

Environmental exposure to methyl mercury (MeHg) results in negative effects on the cardiovascular system, whereas dietary fish oil, rich in n-3 long chain polyunsaturated fatty acids (LCPUFA), decreases the risk of coronary heart disease. Toxicity from mercury is associated with oxidative stress in which mercury induces the generation of reactive oxygen species (ROS) and alters the antioxidant defense systems of cells.

The purpose of this study was to assess interactive effects of MeHg and n-3 LCPUFA on oxidative stress as a potential mechanism for their opposite effect on the cardiovascular system. Specifically, the objective of this study was to assess the potential interaction between MeHg and n-3 LCPUFA on pro- and anti-oxidant enzymes, as well as protein oxidation in heart and liver of animals (n = 8 animals/group) exposed chronically to MeHg and fed a diet with (+) or without (-) n-3 LCPUFA.

The (+) n-3 LCPUFA groups had significantly lower gene expression of the pro-oxidant enzyme (NADPH oxidase) than (-) n-3 LCPUFA groups ($P \leq 0.001$). In addition to the diet effect, a mercury effect was observed for this enzyme in the liver ($P = 0.01$), but not the heart.

The gene expression of glutathione peroxidase decreased with mercury exposure in both the liver and heart tissues. Similarly, methylmercury exposure had a significant effect on the gene expression of catalase but only in the liver. Although the significance level was not reached for a main diet effect, the n-3 LCPUFA 0 MeHg group had the greatest gene expression of GSH-PX in both the heart and liver. Moreover, in the livers of animals not exposed to mercury, the catalase gene expression was greater in the (+) n-3 LCPUFA groups than in those receiving the (-) n-3 LCPUFA diet. Although a similar trend was observed in the heart tissue, the significance level was not reached for catalase gene expression. No significant differences were observed in the gene expression of superoxide dismutase in both liver and heart.

In addition, n-3 LCPUFA-fed animals, in the absence of MeHg exposure, have decreased oxidized protein content in the cardiac tissue by 37% compared to the (-) n-3 LCPUFA group. In the current study the *in vitro* effect of mercury on protein oxidation was compared with iron, a well studied pro-oxidant. Both metals were comparable in their effects on *in vitro* oxidation of bovine serum albumin.

The opposing effect of environmental contaminant, MeHg, and naturally occurring nutrients found in fish, n-3 LCPUFA, may be explained, in part, through changes in oxidative status.

Introduction

Oxidative stress refers to an imbalance between reactive oxygen species (ROS) and antioxidant defenses. Cell damage is induced by ROS which involves the production of superoxide by the coupling of electrons to molecular oxygen, and its subsequent

reduction to hydrogen peroxide and hydroxyl radicals. Oxidative damage indicates the presence of biomolecules altered by the prooxidants such as lipid peroxidation, protein disruption and disintegration, and DNA damage (167). NADPH oxidase family of enzymes is considered the major source of ROS (168) and of vascular superoxide, in particular (169). Under normal conditions, ROS are eliminated from the cell by the action of the antioxidant enzymes: superoxide dismutase (SOD), catalase, or glutathione peroxidase (GSH-PX). Oxidative damage is implicated in pathogenesis of multiple disorders such as cardiovascular disease, insulin resistance and diabetes mellitus (170). Increased production of ROS was found in adipose tissue of obese mice as well as increased expression of pro-oxidant enzyme NADPH oxidase, whereas the mRNA expression of antioxidant enzymes SOD, GSH-PX and catalase was decreased (171).

Toxicity from mercury is associated with oxidative stress in which mercury induces the generation of reactive oxygen species (ROS) and alters the antioxidant defense systems of cells. ROS formation is considered a biomarker of MeHg neurotoxicity (172, 173). *In vitro* and *in vivo* exposure to MeHg increased the rate of formation of ROS and altered the activity of antioxidant enzymes (172, 174-177). Recently, Reardon and Bhat (178) have reviewed the role of oxidative stress in the methylmercury-induced neurotoxicity.

Supplementation with fish oil containing n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) increases antioxidant enzymes activity such as superoxide dismutase (SOD) and decreased lipid peroxidation in animals (179). Green et al. (180) has hypothesized that the neuroprotective effect of n-3 LCPUFA could be due to enhanced free radical scavenging and decreased lipid peroxidation

Even though a relationship between oxidative stress and methylmercury and n-3 LCPUFA, individually, has been established, the interactive effects of mercury and n-3 LCPUFA on the oxidative status of cardiac and hepatic tissue have not been investigated. The objectives of this study were to assess the potential interaction between MeHg and n-3 LCPUFA on the pro- and anti-oxidant enzymes, as well as, protein oxidation in animals exposed chronically to MeHg and fed a diet with or without n-3 LCPUFA.

Methods

Animals, diets and experimental design

Adult Long Evans female rats were exposed from 5 to 18 months of age to 0, 0.5, and 5.0 ppm MeHg in drinking water providing approximately 0, 40 and 400 microgram/kg body weight/day of mercury, and fed either a diet without (-) or a diet with (+) n-3 LCPUFA.

The diet for each group (n = 8 animals/group) was based on the AIN-93 formulation but differed in the composition of the dietary fat with fat mixtures replacing the soybean oil (Research Diets, Inc, New Brunswick, NJ). The (-) n-3 LCPUFA diet contained a mixture of palm, safflower, soybean and coconut oils and the (+) n-3 LCPUFA diet contained the same mixture except that a special EPAX fish oil (Pronova Biocare, Lysaker, Norway) replaced the coconut oil in the mixture. The composition of the oil mixtures is given in Table 7.

Specifically, the fat mixture for the (-) n-3 LCPUFA diet consisted of 42.8% palm oil, 9.2% safflower oil, 15.0% soybean oil and 33% coconut oil, whereas the (+)n-3 LCPUFA diet contained 33% fish oil instead of coconut oil. The mixture of oils was used

in order to keep the levels of linoleic (18:2n-6) and alpha-linolenic (18:3n-3) acid relatively constant (Table 8). The n-6 fatty acid as well as the monounsaturated fatty acids content was almost equal in the two diets. Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) were provided in the (+) n-3 LCPUFA diet at 5% each of total fatty acids. The ratios of total n-6 to n-3 PUFA were 20 for the (-)n-3 LCPUFA diet and 1.8 for the (+)n-3 LCPUFA diet .

Both diets had the same mineral and vitamin content. Thus, the nutrient composition of the two diets differs only in the dietary fat type. In order to minimize the oxidation, the oil mixtures were supplemented with an antioxidant mixture of tocopherols Coviox T-70 (10 g/25 kg oil) and Copheral F (14 g/25 kg oil). T-butylhydroquinone (TBHQ) was used as per the AIN-93 formulation in both diets. The pelleted diet was kept in a freezer and provided daily.

The animals were exposed to mercury by adding methylmercuric chloride (Alfa Aesar, Ward Hill, MA) to the drinking water. Sodium carbonate (< 5 nmolar) was used to buffer all drinking water. At 18 months of age, the animals were anesthetized, euthanized, and the heart and liver removed. The tissue was weighed, blanketed with nitrogen, quick frozen in liquid nitrogen, and stored at -80^0 C for later analysis. All animals were housed in a temperature and humidity- controlled facility with a 12-h light/dark cycle. The Auburn University Animal Care and Use Committee approved the protocol. The colony was housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility.

Pro-oxidant/antioxidant enzymes

The oxidant system enzymes were investigated by determining the gene expression levels of pro-oxidant enzyme (NADPH oxidase) and antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase) in addition to the catalase enzyme activity.

RNA isolation and real-time PCR analysis

Total RNA was isolated from 35 mg of rat cardiac and hepatic tissue with on-column DNase digestion using an RNeasy® Mini Kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using an iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA). Quantitative real-time PCR was performed on an iCycler real-time PCR detection system (BioRad, Hercules, CA) using 0.5 µl cDNA, 3.0 µl of a 1.25 µM (final concentration) primer mix, and 12.5 µl of iQ SYBR Green Supermix per reaction (BioRad, Hercules, CA). Rat gene specific primers were selected from Primer Bank (http://pga.mgh.harvard.edu/primerbank/citation.html), and purchased from Integrated DNA Technologies, Inc. (IDT, Inc., Coralville, IA). The following primers (5'-3') were used: rat NADPH oxidase, NM_023965, CTGCAGCCTGCCTGAATTTCAACT (forward) and CAATGGTGTGAATGGCCGTGTGAA (reverse); rat catalase, NM_012520, TGGAGAGGCAGTGTACTGCAAGTT (forward) and TAATCCGGGTCTTCCTGTGCAAGT (reverse); rat glutathione peroxidase 1, NM_022525, TCACACTTTCTCCAGCTTCCCGTT (forward) and TCATGTGGGCATATGGGAGATGCT (reverse); rat superoxide dismutase 1, NM_017050, GGTGTGGCCAATGTGTCCATTGAA (forward) and CAATCCCAATCACACCACAAGCCA (reverse); rat β-actin, NM_031144,

TTGCTGACAGGATGCAGAAGGAGA (forward) and ACTCCTGCTTGCTGATCCACATCT (reverse); rat cyclophilin, NM_022536, TGAGCGCTTCCCAGATGAGAACTT (forward) and CATGCCTTCCAGAACTTTGCCGAA (reverse). The analysis was performed in triplicate.

Expression levels were normalized to β-actin for the liver and cyclophilin for the heart using the ΔCt method as described previously (181). ΔCt value (ΔCt = Gene Ct – Housekeeping Gene Ct) was used for statistical analyses, and reported as relative gene expression.

Catalase activity

Catalase activity was assayed colorimetrically by the method of Sinha (182). Catalase causes rapid transformation of hydrogen peroxide (H_2O_2) to H_2O. The method is based on the principle that dichromate/acetic acid reagent is reduced to chromic acetate when heated in the presence of hydrogen peroxide. The chromic acetate was measured colorimetrically at 610 nm. The reaction mixture consisted of 1 mL phosphate buffer (pH 7.0), 0.2 mL of the diluted liver protein lysate and 0.5 mL of 0.2 M H_2O_2. 2 mL of dichromate/acetic acid reagent (5% solution of potassium dichromate in water with glacial acetic acid in the ratio of 1:3 and diluted 1:5 with water) was added after 2 min of incubation. The H_2O_2 was added after the addition of the dichromate/acetic acid reagent to the control tube which was considered ‘zero time’. The tubes were boiled for 10 min, cooled and the absorbance was read at 610 nm. The assay was conducted in duplicate. The activity of catalase was expressed as μmoles of H_2O_2 consumed/min/mg protein.

In vivo protein oxidation

Protein oxidation was assessed in the heart and liver tissues by measuring protein carbonyl levels using Western blot procedures according to the manufacturer's instructions (Oxyblot; Chemicon, Temecula, CA, USA). Heart and liver proteins (35-40 mg) were isolated using lysis buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Tx-100, 1 Complete Mini protease inhibitor cocktail tablet (Roche Diagnostic, Manheim, Germany). Protein concentrations were determined using Bio-Rad protein assay kit (BioRad, Hercules, CA). Carbonyl groups were derivatized to 2, 4 dinitrophenylhydrazone (DNP-hydrazone) by reaction with dinitrophenylhydrazine (DNPH). The derivatized samples were separated by gel electrophoresis followed by Western blotting. The membrane was incubated with primary antibody specific to the DNP followed by incubation with a horseradish peroxidase-antibody conjugated against the primary antibody. The membrane was then treated with chemiluminescent reagents (ECL Plus, Amersham Biosciences). Light emission was detected with a highly sensitive imaging system (UVP BioImaging System, Upland, CA). Signals were quantified using EpiChemi^3 Darkroom and LabWork software (Ultra-violet Products Ltd., Upland, CA) and normalized to β-actin.

In vitro pro-oxidant effect of mercury

Bovine serum albumin (BSA) (Fisher Scientific, Fair Lawn, NJ) was used to compare the effect of mercury with iron. 5 mg/mL of protein sample was prepared in the following buffer: 25 mM HEPES buffer, pH 7.2; 25 mM ascorbate; and either 100 μM ferric chloride or 100 μM methylmercuric chloride. The ‘zero time’ sample was not

exposed to Fe or Hg. BSA samples were incubated at 37^0C for 1 hr and 5 hr. Samples were dialyzed overnight vs. 50 mM HEPES buffer, 1 mM EDTA. Protein concentrations were determined again at the end of dialysis. Mercury-induced oxidation of BSA was investigated by measuring protein carbonyl levels as described above.

Statistics

Two-way ANOVA with post hoc Tukey-Kramer adjustment and least significance difference analysis for multiple comparisons were performed to test for differences in gene expression of enzymes and catalase activity among all six experimental groups. Dunnett's test was used to determine differences in protein oxidation among groups. Results were expressed as means ± SE and statistical significance was set at $P \leq 0.05$. Statistical analysis was performed using the SAS 9.1 statistical software (SAS Institute Inc., Cary, NC).

Results

Pro-oxidant/antioxidant enzymes

The 2 (dietary treatment) x 3 (methylmercury levels) factorial design allowed the investigation of diet and MeHg main effects, as well as, their possible interaction. The cardiac and hepatic gene expression levels of the pro-oxidative and antioxidant enzyme are shown in Table 12.

A dietary effect was obtained for the gene expression of the pro-oxidant enzyme, NADPH oxidase in heart and liver. The (+) n-3 LCPUFA groups had significantly lower gene expression of NADPH oxidase than (-) n-3 LCPUFA groups. In addition to the diet

effect, a mercury effect was observed for this enzyme in the liver, but not the heart. The 5 ppm MeHg exposure group without n-3 LCPUFA had the greatest gene expression of NADPH oxidase.

Mercury exposure also altered the gene expression of the anti-oxidant enzymes (Table 12). For example, a significant mercury effect on expression of GSH-PX was obtained in both the heart and liver tissues. The gene expression of GSH-PX decreased with mercury exposure. Similarly, methylmercury exposure had a significant effect on the gene expression of catalase but only in the liver. No effect of methylmercury was found on gene expression of superoxide dismutase (SOD).

In contrast, a statistically significant effect of diet was not observed on any of the antioxidant enzymes studied. Although the significance level was not reached for a main diet effect, the n-3 LCPUFA 0 MeHg group had the greatest gene expression of GSH-PX in both the heart and liver. Moreover, in the livers of animals not exposed to mercury, the catalase gene expression was greater in the (+) n-3 LCPUFA groups than in those receiving the (-) n-3 LCPUFA diet. Although a similar trend was observed in the heart tissue, the significance level was not reached. No significant differences in the expression of SOD were observed in the cardiac or hepatic tissues.

Although no significant difference was observed, the (+) n-3 LCPUFA no mercury group had greater catalase activity than (-) n-3 LCPUFA groups, reflective of the same pattern observed in gene expression (Table 13).

***In vivo* protein oxidation**

The results of the protein oxidation assays of the heart and liver are represented in Figures 2 and 3. The (-)n-3 LCPUFA 0 MeHg group was considered as a reference group

in determining the level of oxidized proteins. The (+) n-3 LCPUFA 0 MeHg group had a 37 % reduction in cardiac protein oxidation compared with (-) n-3 LCPUFA group. No difference was observed in groups exposed to MeHg. Although a similar trend was observed in the liver, the significance level was not reached.

***In vitro* pro-oxidant effect of mercury**

The protein oxidation induced by mercury or iron was assessed by comparison to 'zero time' point which was considered to be 100 % (Figure 4). Both iron and mercury induced BSA oxidation at 1 hr and 5 hr time points in a similar manner.

Discussion

Oxidative damage is caused by an imbalance in the oxidative defense mechanisms: an increased in the pro-oxidant enzymes, or a decrease in the anti-oxidant enzymes. This is the first study in which the interactive effects of n-3 LCPUFA and mercury on the expression levels of both the pro- and anti-oxidant enzymes were investigated. The gene expression of the pro-oxidant enzyme, NADPH oxidase, and of the antioxidant enzymes, superoxide dismutase (SOD), catalase, or glutathione peroxidase (GSH-PX) were determined by RT-PCR.

In the current study, pro-oxidant enzyme gene expression (NADPH oxidase) was increased by methylmercury exposure and decreased by n-3 LCPUFA. The effect of mercury reached statistical significance only in the liver, while the effect of n-3 LCPUFA in decreasing the expression of NADPH oxidase was clearly evident in both the heart and liver. Thus, n-3 LCPUFA and MeHg exerted an opposing effect on pro-oxidant enzyme gene expression.

The most beneficial scenario would be for n-3 LCPUFA to increase antioxidant enzymes that had been decreased by MeHg exposure. Although there was some indication that MeHg decreased antioxidant enzymes, particularly hepatic enzymes for animals given n-3 LCPUFA, the effect was not clear-cut. However, it was clear that animals not exposed to MeHg and supplemented with n-3 LCPUFA demonstrated the greatest gene expression of antioxidant enzymes. This was significant ($p < 0.05$) for glutathione peroxidase in heart and glutathione peroxidase and catalase in liver. It is interesting to note that this was the only group [0 MeHg, (+) n-3 LCPUFA] that did not exhibit a rise in blood pressure with age (119).

Other investigators have reported a similar increased gene expression of antioxidant enzymes with dietary fish oil. For example, Venkatraman et al. (183) and Takahashi et al. (184) reported that livers of mice fed a fish oil diet containing n-3 LCPUFA demonstrated significantly decreased gene expression of glutathione transferase, uncoupling protein 2, superoxide dismutase (183), and catalase, glutathione peroxidase, superoxide dismutase (184).

An increase in activity as well as gene expression has also been observed by a number of investigators (179, 183, 185-191). In all of these studies, greater activity of antioxidant enzymes e.g. GSH-PX, catalase, SOD was documented in animals fed fish oil diet in comparison with animals fed control diets.

Methylmercury-induced changes in the antioxidant enzymes activity have been previously investigated. Watanabe et al. (192) found a decreased in GSH-PX activity induced by MeHg in the fetal mouse brain. Prenatal exposure to MeHg has been found to decrease the levels of GSH-PX, catalase and SOD activities in hippocampus of rats (177).

Exposure to 10-40 mg/L MeHg in drinking water for 2 weeks results in decreased activity of GSH-PX in kidney of mice (193). Glutathione peroxidase reduces peroxides, thus by decreasing its activity, the oxidative stress may increase.

Long treatment (10 days) with low concentrations of methylmercury (nanomolar) increases the content of ROS while the activities of the antioxidant enzymes (catalase and GSH-PX) decrease in rat brain cell culture (176). The animals from the present study were exposed chronically to relatively low to medium concentration of methylmercury (0.5 ppm or 5 ppm) (141). The highest mercury dose used resulted in an exposure of approximately 400 μg/kg/day which is 2.5 orders of magnitude greater than the current reference dose for MeHg (0.1 μg/kg/day) established by the United States Environmental Protection Agency (141-143). The exposure level in rat is greater than the primate exposure due to the greater density of red blood cells to which mercury is known to bind.

In the current study the *in vitro* effect of mercury on protein oxidation was compared with iron, a well studied pro-oxidant. Both metals were comparable in their effects on *in vitro* oxidation of bovine serum albumin. Several mechanisms for iron induction of oxidation have been suggested. Iron may enhance oxidation by catalyzing the formation of hydroxyl radicals, decomposition of lipid hydroperoxides and formation of free radicals (194). Iron overload may also elicit antioxidant defenses involving thiol metabolism which play a role in resistance to iron-induced oxidative damage (195). Increase in hepatic glutathione (GSH) levels and induction of enzymes involved in GSH metabolism were found with iron overload (196, 197).

Whether mercury acts by similar mechanisms is not known. Exposure to MeHg *in vitro* (198) and *in vivo* (193, 199) is known to increase lipid peroxidation. Parenteral administration of 5 mg/kg MeHg enhanced hepatic lipid peroxidation of rats (199).

Addition of antioxidants may protect against oxidation induced by mercury. For example, reduced gluthatione, vitamin E, and selenite protect astrocytes and neuroblastoma cells from MeHg neurotoxicity (200). However, vitamin E only partially alleviates MeHg neurotoxicity *in vivo* (201). The interaction between blood and brain mercury and selenium concentration after developmental and chronic exposure to methylmercury was investigated by Newland et al. (140).

In addition, MeHg increases the rate of formation of ROS (177); ROS being considered potent mediators of MeHg cytotoxicity (173, 174). ROS production may be a mechanism which triggers intracellular Ca^{2+} rise in MeHg neurotoxicity (202). The exact mechanism of mercury-induced ROS production is not fully understood. Methylmercury has great affinity for thiols which causes a decrease in intracellular glutathione levels and accumulation of ROS (203). Moreover, a common pathway in ROS formation between iron and MeHg may be present. Pretreatment with deferoxamine, a potent iron-chelator, prevented MeHg-induced increase of ROS in synaptosomes isolated from untreated rats (172).

Numerous studies have investigated the negative effects of MeHg on lipid peroxidation, antioxidants (glutathione imbalances) and DNA and mitochondrial membrane damage (175, 192, 193, 199, 204-206). However, there is a lack of studies that investigate mercury-induced changes in proteins. The toxic effects of MeHg on proteins

need to be investigated due to the fact that MeHg is mostly bound to sulfur-containing proteins.

The interactive effects of MeHg and n-3 LCPUFA on protein oxidation was investigated in the present study by measuring protein carbonyl levels in the rat heart and liver tissues. The n-3 LCPUFA-fed animals in the absence of MeHg exposure have decreased oxidized protein in cardiac tissue compared to the (-) n-3 LCPUFA group or mercury exposed animals.

It is important to note the similarity of decreasing protein oxidation in (+) n-3 LCPUFA 0 MeHg group with the changes in the blood pressure of the same animals (119). Significantly low blood pressure was observed only in the absence of MeHg and presence of n-3 LCPUFA. Similarly, the protein oxidation was lower in the (+) n-3 LCPUFA 0 MeHg group compared to all the other groups. Therefore, the oxidative damage status assessed by the protein oxidation levels in the cardiac tissue may explain, in part, the previously observed changes in the blood pressure. On the other hand, the protective role of n-3 LCPUFA in the development of hypertension may involve other mechanisms such as reducing the production of inflammatory mediators, acting as precursors of the 3-series prostaglandins which are antiaggregators and vasodilators (153), and changes in the membrane fatty acids composition (see Chapter III).

Imbalance in antioxidant status has been associated with essential hypertension (207). Reduced production of vasodilator nitric oxide or increased production of superoxide anion promotes endothelial dysfunction, induces vasoconstriction and high blood pressure (208). NADPH oxidase represents the predominant source of superoxide anion in endothelium. Competitive inhibition of NADPH oxidase completely inhibits

superoxide anion production and alleviated increased blood pressure (209). NADPH oxidase is even considered a general mechanism by which high blood pressure develops, irrespective of the mode of induction of hypertension (210). The essential n-3 fatty acids have been found to decrease superoxide anions in the myocardium and coronary arteries in monkeys (211). Moreover, n-3 fatty acids suppress the production of ROS by 30-55% in stimulated leukocytes (212-214). Although the alleviating effect of n-3 LCPUFA on ROS production is well known, the present study is the first to investigate the direct action of n-3 LCPUFA on NADPH oxidase gene expression.

Previously, we have found that dietary fish oil rich in n-3 LCPUFA caused a decrease in blood pressure and prevented the rise in blood pressure with age in rats (119). In the present study, we have shown that dietary n-3 LCPUFA decreased the gene expression of the pro-oxidant enzyme (NADPH oxidase) in the heart and liver of same animals, whereas MeHg increased the hepatic expression of this enzyme. Antioxidant capacity was greatest in animals fed (+) n-3 LCPUFA diet and unexposed to MeHg. Therefore, based on the findings of this study, naturally occurring nutrients found in fish, such as n-3 LCPUFA, may exert their beneficial effects on blood pressure, in part, through changes in oxidative status.

TABLE 7
Nutrient composition of experimental diets

	(-) n-3 LCPUFA diet	(+) n-3 LCPUFA diet
	g/ 100 g diet	
Protein	14.3	14.3
Carbohydrate	73.1	73.1
Fat	4.0	4.0
kcal/gm	3.9	3.9
Ingredient	g	
Casein, 30 Mesh	140.0	140.0
L-Cystine	1.8	1.8
L-Methionine	0.8	0.8
Corn Starch	496.012	496.012
Maltodextrin	125.0	125.0
Sucrose	100.0	100.0
Cellulose	50.0	50.0
EPAX Fish Oil [1]	0.0	13.2
Coconut Oil [2]	13.2	0.0
Palm oil	17.12	17.12
Safflower Oil	3.68	3.68
Soybean Oil	6.0	6.0
t-Butylhydroquinone	0.008	0.008
Mineral mix [3]	35.0	35.0
Vitamin mix [4]	10.0	10.0
Choline Chloride	1.38	1.38
Total	1000	1000

[1] AIN-93 Diet for Mature Rodents containing a blend of fish, palm, safflower, and soybean oils (Research Diets, Inc, New Brunswick, NJ).
[2] AIN-93 Diet for Mature Rodents containing a blend of coconut, palm, safflower, and soybean oils (Research Diets, Inc, New Brunswick, NJ).
[3] Supplied per kg of mix: calcium (357 g); copper (0.3 g); iodine (10 mg); iron (6.06 g); magnesium (24 g); manganese (0.63 g); potassium (324.6 g); selenium (10.25 mg); zinc (1.65 g).
[4] Supplied per kg of mix: retinyl palmitate (0.8 g); vitamin D (75 mg); vitamin E (15 g); vitamin thiamin (0.6 g); riboflavin (0.6 mg); pyridoxine (0.7 g); B12 (2.5g); calcium pantothenate (1.6 g); nicotinic acid (3 g); biotin (20mg); folic acid (0.2 g).

TABLE 8

Fatty acid composition of experimental diets

Fatty acid	(-) n-3 LCPUFA[1]	(+) n-3 LCPUFA[2]
	% by weight	
10:0	0.8	0.1
12:0	17.9	1.5
14:0	8.1	3.7
16:0	25.6	29.2
18:0	4.1	4.6
16:1 n-7	0.2	2.5
18:1[3]	22.5	25.9
18:2 n-6	20.0	21.2
18:3 n-3	1.0	1.3
20:5 n-3	-	5.0
22:6 n-3	-	5.2
∑ SFA[4]	56.5	39.1
∑ MUFA[4]	22.7	28.4
∑ PUFA[4]	21.0	32.7
∑ n-3	1.0	11.5
∑ n-6	20.0	21.2
∑ n-6/∑ n-3	20.0	1.8
∑ n-3/∑ n-6	0.05	0.5

[1] Mixture of coconut, palm, safflower, and soybean oils.
[2] Mixture of fish, palm, safflower, and soybean oils.
[3] 18:1 n-9 + n-7.
[4] Abbreviations as follows: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long-chain polyunsaturated fatty acids.

TABLE 12

Cardiac and hepatic gene expression of the oxidative system enzymes[1]

A. Heart		Pro-oxidant		Anti-oxidant	
Diet	MeHg	NADPH oxidase	Glutathione Peroxidase	Catalase	Superoxide dismutase
(-) n-3	0 MeHg	4.45 ± 0.5 ^{c}	1.90 ± 0.1 ^{ab}	1.33 ± 0.1	7.94 ± 1
LCPUFA	0.5 MeHg	4.13 ± 0.5 ^{bc}	1.48 ± 0.1 ^{a}	1.28 ± 0.1	8.14 ± 1
	5 MeHg	4.49 ± 0.5 ^{c}	1.80 ± 0.1 ^{ab}	1.21 ± 0.1	7.79 ± 1
(+) n-3	0 MeHg	2.36 ± 0.5 ^{a}	1.96 ± 0.1 ^{b}	1.65 ± 0.1	9.45 ± 1
LCPUFA	0.5 MeHg	2.73 ± 0.5 ^{ab}	1.60 ± 0.1 ^{ab}	1.55 ± 0.1	9.01 ± 1
	5 MeHg	3.12 ± 0.5 ^{ab}	1.90 ± 0.1 ^{ab}	1.20 ± 0.1	9.42 ± 1
MeHg effect	P value	NS	0.04	NS	NS
Diet effect	P value	0.0002	NS	NS	NS

B. Liver		Pro-oxidant		Anti-oxidant	
Diet	MeHg	NADPH oxidase	Glutathione Peroxidase	Catalase	Superoxide dismutase
(-) n-3	0 MeHg	0.60 ± 0.1 ^{b}	0.96 ± 0.2 ^{a}	0.83 ± 0.1 ^{a}	2.44 ± 0.3
LCPUFA	0.5 MeHg	0.66 ± 0.1 ^{b}	1.04 ± 0.2 ^{ab}	0.80 ± 0.1 ^{a}	2.61 ± 0.3
	5 MeHg	1.07 ± 0.1 ^{c}	0.80 ± 0.2 ^{a}	0.70 ± 0.1 ^{a}	2.24 ± 0.3
(+) n-3	0 MeHg	0.29 ± 0.1 ^{a}	1.45 ± 0.2 ^{b}	1.25 ± 0.1 ^{b}	2.46 ± 0.3
LCPUFA	0.5 MeHg	0.46 ± 0.1 ^{ab}	0.85 ± 0.2 ^{a}	0.85 ± 0.1 ^{a}	2.63 ± 0.3
	5 MeHg	0.56 ± 0.1 ^{ab}	0.76 ± 0.2 ^{a}	0.66 ± 0.1 ^{a}	2.72 ± 0.3
MeHg effect	P value	0.01	0.05	0.005	NS
Diet effect	P value	0.001	NS	NS	NS

[1] Data expressed as relative mRNA levels (means ± SE) (n = 8/group). Two-way ANOVA was used to determine diet and MeHg main effects. Values in a column with different superscript are significantly different ($P \leq 0.05$) as determined by post hoc LSD analysis. No significant interactions between diet and MeHg by two-way ANOVA were observed for any parameter.

TABLE 13
Hepatic catalase activity[1]

(-) n-3LCPUFA			(+) n-3LCPUFA		
0 MeHg	0.5 MeHg	5 MeHg	0 MeHg	0.5 MeHg	5 MeHg
2.56 ± 0.2	2.57 ± 0.2	2.63 ± 0.2	2.89 ± 0.2	2.69 ± 0.2	2.86 ± 0.2

[1] Data expressed as μmoles H_2O_2 consumed/min/mg protein (means ± SE) (n = 8/group). Catalase was assayed colorimetrically by the method of Sinha (182). No significant difference was observed by two-way ANOVA.

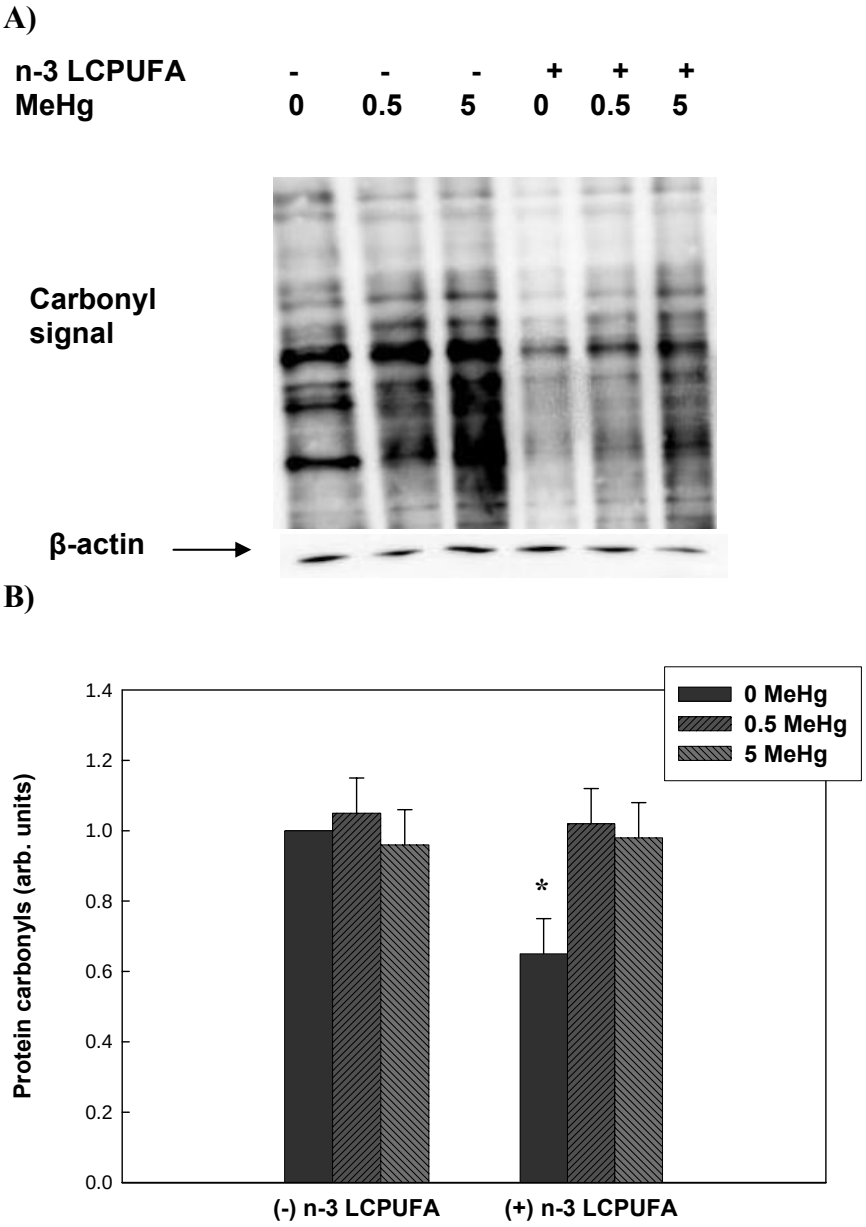

FIGURE 2. Cardiac protein oxidation. A) Carbonyl signal obtained by Western blot B) Quantitative analysis of carbonyl signal normalized for protein loading. The (-)n-3 LCPUFA 0 MeHg group was considered as reference group. The carbonyls were detected by reaction with 2,4-Dinitrophenylhydrazine (DNPH) and anti-DNPH immunostaining. (*), indicate significant difference compared to reference group ($P \leq 0.05$) by ANOVA followed by Dunnett's test (n = 8/group).

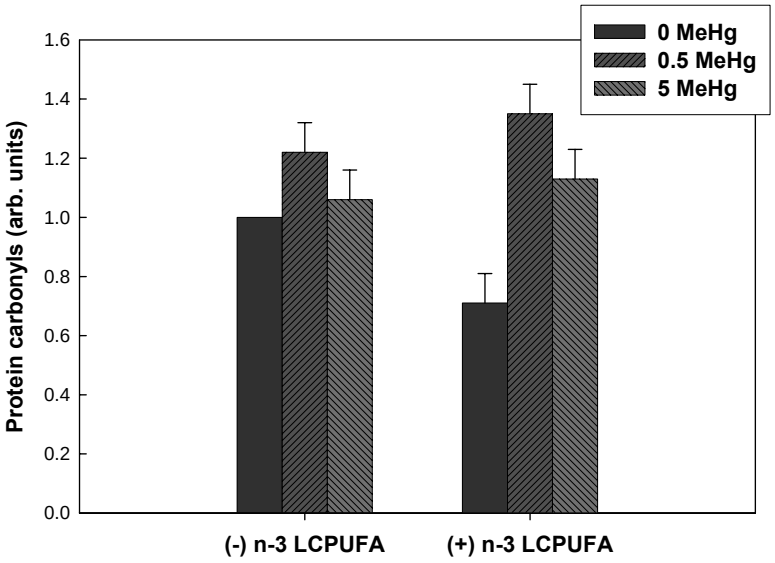

FIGURE 3. Hepatic protein oxidation. The (-) n-3 LCPUFA 0 MeHg group was considered as reference group. The carbonyls were detected by reaction with 2,4-Dinitrophenylhydrazine (DNPH) and anti-DNPH immunostaining. No significant differences were observed using ANOVA followed by Dunnett's test (n = 8/group).

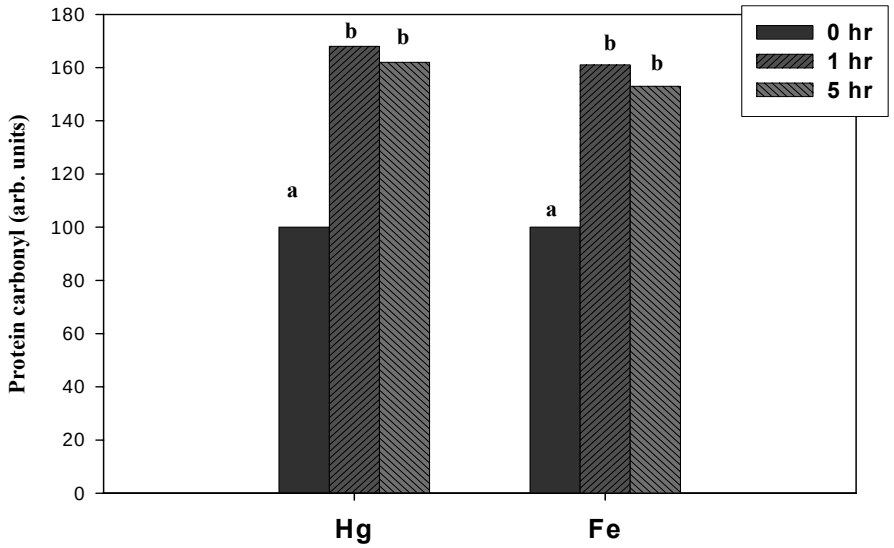

FIGURE 4. *In vitro* oxidation of Bovine Serum Albumin. Protein sample was incubated with 100 μM ferric chloride or 100 μM methylmercuric chloride at 37^0C for 1 hr and 5 hr. The 'zero time' sample was not exposed to Fe or Hg. The carbonyls were detected by reaction with 2,4-Dinitrophenylhydrazine (DNPH) and anti-DNPH immunostaining. Different letters on the bars indicate significant differences among different groups ($p \leq 0.05$) by ANOVA followed by Dunnett's test.

CHAPTER V. CONCLUSIONS

Overall conclusions

The beneficial health effects of n-3 long chain polyunsaturated fatty acids in fish on human health and the cardiovascular system in particular, is well known. However, the mechanisms of action of these fatty acids are not clear yet. The focus of the present research study was to investigate the cardiovascular protective effects of n-3 LCPUFA in relationship to the environmental contaminant, methylmercury.

Although methylmercury and long chain n-3 polyunsaturated fatty acids have similar sources (fish and seafood products), they exert opposite effects on the cardiovascular system: n-3 LCPUFA decrease blood pressure and the risk of coronary heart disease, and promote an antiarrhythmic effect; MeHg increases blood pressure, causes irregular heart rate and tachycardia. Thus, a potential interaction between them has been proposed in this research study. In a previous study, we have shown that animals consuming a diet containing n-3 LCPUFA exhibited significantly lower blood pressure compared to control animals, in the absence of MeHg. In addition, dietary n-3 LCPUFA prevented the increase in blood pressure with age only in the absence of MeHg exposure.

The opposite effects of dietary n-3 LCPUFA and MeHg on blood pressure could be explained in part, through two mechanisms which were investigated in this research project:

1) Changes in the fatty acid profile of cardiac phospholipids.

The objective of this project was to determine if chronic exposure to MeHg alters the heart phospholipid fatty acid profile of the adult female rat, and if dietary n-3 LCPUFA could help overcome the adverse effects of mercury.

High incorporation of DHA was obtained with dietary n-3 LCPUFA supplementation and the n-6 unsaturated fatty acids content was decreased in both phosphatidylethanolamine (PE) and phosphatidylcholine (PE) fractions of the heart. Dietary DHA incorporation into myocardial membrane has been shown to reduce the incidence and severity of ventricular arrhythmias (161). Modification of membrane fatty acid composition may be one of the mechanisms by which n-3 LCPUFA have beneficial effects on the cardiovascular system. Methylmercury did not affect DHA (22:6n-3) and other essential fatty acids in heart PE and PC fractions. Thus, dietary n-3 LCPUFA had profound effects on the cardiac phospholipid fatty acid composition in a beneficial manner irrespective of methylmercury exposure.

2) Changes in the oxidative status.

There were two objectives of this study: a) to assess the potential interaction between MeHg and n-3 LCPUFA on pro-oxidant/antioxidant status of rat dams exposed chronically to MeHg and fed a diet with or without n-3 LCPUFA; b) to compare the *in vitro* pro-oxidant effect of MeHg to iron.

Dietary n-3 LCPUFA decreased the gene expression of the pro-oxidant enzyme (NADPH oxidase) ($P \leq 0.001$) in both heart and liver, whereas MeHg increased the expression of this enzyme ($P = 0.01$) only in the liver. Animals not exposed to MeHg and supplemented with n-3 LCPUFA demonstrated the greatest gene expression of

antioxidant enzymes (glutathione peroxidase and catalase). Dietary n-3 LCPUFA, in the absence of MeHg, decreased cardiac protein carbonyl content by 37% compared to (-) n-3 LCPUFA diet. The *in vitro* pro-oxidant effect of mercury was comparable to iron, a well studied pro-oxidant.

The n-3 LCPUFA may enhance resistance to oxidative stress through reduction in tissue protein oxidation and pro-oxidant enzyme gene expression, as well as, high gene expression of enzymes of the antioxidant mechanism system. Although the opposing effects between the antioxidant properties of n-3 LCPUFA and the oxidative stress induced by MeHg may explain the cardiovascular effects, other mechanisms still may be in play.

In conclusion, the beneficial effect of n-3 LCPUFA on the gene expression of enzymes from the oxidant system may enhance the efficiency of the antioxidant system which, in addition to the changes in cardiac fatty acid composition may yield a benefit in the cardiovascular disease. Therefore, the balance between the risks and benefits of fish consumption may be obtained by intake of fish rich in n-3 LCPUFA but low in MeHg.

Fish consumption and public health implications

Although the health benefits of n-3 fatty acids from fish are well known, the risks from environmental toxicants, such as methylmercury, create a challenging paradox for consumers. Restriction of fish consumption could negatively affect general population well-being and child development. Oken et al. (215) reported a reduced consumption of

dark meat fish, canned tuna, and white meat fish in a cohort of 2,235 pregnant women after the January 2001 federal advisory recommendation.

The risk and benefit of fish consumption to fetus was examined by Sakamoto et al. (216) by investigating the relationships between red blood cell mercury (RBC-Hg) and plasma fatty acid composition in mother and fetus at parturition. The study included sixty-three healthy Japanese pregnant women and venous blood samples collected from mothers and their fetuses (umbilical cord). There were significant correlations in the EPA and DHA concentrations between maternal and fetal plasma. Fetal RBC-Hg levels were greater than maternal RBC-Hg levels, and a significant correlation was found between RBC-Hg and plasma DHA in the fetus. These findings suggest that methylmercury (MeHg) and DHA originating from maternal fish consumption were taken into maternal circulation and transferred to the fetal circulation. The authors proposed that maternal consumption of fish low in MeHg but rich in DHA would have beneficial effects on children's health.

The Kuopio Ischemic Heart Disease Risk Factor Study, conducted in Finland, showed that high intake of nonfatty freshwater fish (30 g/day) produced an increase in mercury accumulation that was correlated with an increased risk of myocardial infarction, coronary and cardiovascular death (110). On the other hand, the Health Professionals Follow-up Study conducted in US revealed no association between high mercury levels and increased risk of coronary heart disease (94).

The type of seafood consumed with different mercury content can explain the difference in findings from these studies. In the Seychelles, the seafood consumed has lower mercury concentrations (0.3 μg/g) compared with populations from Faroe Islands

or New Zealand (1.6 – 2.2 μg/g). Thus, various concentrations of mercury in the food consumed cause different outcomes. A population-based prospective cohort study conducted in 4 communities in the United States in 1989 and 1990 demonstrated that cardiac benefits of fish consumption on the development of ischaemic heart disease depends on the type of fish meal consumed (217). The risk of coronary heart disease was reduced by the consumption of 1 to 2 servings a week of broiled tuna or baked fish.

The United States Environmental Protection Agency (EPA) set the reference dose of 0.1 μg/kg body weight) for methylmercury ingestion (218). One or two meals per week of some species of fish can result in mercury ingestion exceeding the reference dose depending of the type of fish consumed. Consumption of fish is highly variable across the United States population: inclusion of fish in the diet varied with geographic location, seasons of the year, ethnicity, and personal food preferences (92). It was reported that 88% of all adults in the United States consume fish and shellfish at least once a month with 58% of adults consuming fish at least once a week. Between 13% and 23% consume fish/shellfish two or three times per week, and an estimated 3% indicate they consume fish and shellfish six times a week. Only 1% of all respondents indicated they ate fish and shellfish daily. Thus, methylmercury exposures are considerably less than the reference dose. However, type of fish consumed and greater than average intake correspond to greater MeHg intakes. Annual seafood consumption projections for the U.S. population indicate that 75-93% of adult women and 58-72% of children 2-5 years of age consume seafood (219).

Prenatal methylmercury (MeHg) exposure from fish consumption has become a concern because the developing brain of the fetus is particularly vulnerable. High

gestational exposures were reported with maternal consumption of highly contaminated fish (10-30 ppm mercury) from industrially polluted Minimata Bay, Japan, in the 1950s, or of contaminated grain in Iraq in 1971 (maternal intake, 710-5700 μg/kg per day; 18-598 ppm mercury in maternal hair). However, MeHg exposures are lower among US women of childbearing age, median (10th-95th percentiles) levels of mercury in hair were 0.19 (0.04-1.73) ppm overall and 0.34 (0.09- 2.75) ppm among women consuming >3 servings of fish per month (220). The total hair Hg levels of NHANES children and women were generally lower than the levels reported in other studies of U.S. and international populations.

Two large epidemiological studies have been carried out on fish eating population chronically exposed to MeHg (9, 12). Impaired cognitive function was found to be associated with prenatal exposure to MeHg in the Faroe Island (9) but not in the Seychells (12). Differences in the outcome of the studies that investigated mercury toxicity may be due to the presence of beneficial compounds in fish such as n-3 LCPUFA.

Mahaffey (100) has reviewed the methylmercury and n-3 fatty acids composition of several fish and shellfish species and observed that several species low in methylmercury is rich in n-3 fatty acids and vice versa. Shellfish are low in Hg (0.08 ppm) but provide a good source of n-3 fatty acids (0.6 g EPA + DHA/100g tissue). Mackerel, herring and several species of salmon are rich in n-3 fatty acids providing an average of 1.5 g EPA + DHA/100g edible tissue. In contrast, there are four fish species (swordfish, tilefish, shark, and king mackerel) on the federal Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) joint advisory due to

their high Hg content (≥ 1 ppm). These four species are not good sources of n-3 fatty acids, providing only about 0.3 g EPA + DHA/ 100 g edible tissue (143).

The American Heart Association has recommended that adults should consume fish at least twice a week in order to promote cardiovascular health (221). In 2004, the FDA and EPA made 3 recommendations for women who may become pregnant, pregnant women, nursing mothers and young children: to avoid consumption of high mercury containing fish and shellfish such as shark, swordfish, king mackerel, tilefish; to limit their intake of fish to 12 ounces a week; and to check local advisories about the safety of fish caught and if these are not available, to consume only 6 ounces a week of fish caught. It was also specified that canned light tuna has lower mercury level than albacore ("white") tuna. The fish considered low in mercury includes: shrimp, canned light tuna, salmon, pollock, and catfish. For young children smaller portions compared to adults have been recommended (143).

In conclusion, consumption of fish low in MeHg but rich in n-3 LCPUFA would have beneficial effects on the health of children and general population. Concentrations of MeHg in fish depend on the environmental contamination and the predatory species and lifespan of the fish species. Environmental contamination needs to be assessed and accordingly adjust the national advisory of fish consumption. It is recommended to check the local advisory for MeHg content of locally caught fish (143). Larger, longer-living predators such as swordfish and shark have higher MeHg tissue concentration compared to smaller and shorter-living species such as, shellfish, canned light tuna, catfish, and salmon.

Beneficial effects on the cardiovascular system were observed at intakes of fatty fish of 60 g /day (117) or 1-2 servings /week (42-44) which correspond approximately to 6-12 oz/week. For example, 100g of salmon provide about 1.5 g EPA + DHA and contain < 0.1 ppm Hg. These fish intake recommendations may supply low n-3 LCPUFA intake for persons with cardiovascular disease. Prospective randomized trials have shown that daily supplementation with 1-1.8 g EPA+ DHA have beneficial effects on cardiovascular system (46-49). Higher n-3 LCPUFA intake for persons with cardiovascular disease may be achieved by consuming fish richest in n-3 LCPUFA and fish oil supplements (143). The mercury level in fish oil supplements is considered to be negligible (222). The 2007 guidelines for cardiovascular disease prevention in women recommend n-3 fatty acids in capsule form (approximately 850-100 mg of EPA and DHA) to women with coronary heart disease and higher doses (2-4 g) may be used for treatment of hypertriglyceridemia (122).

Therefore, based on the current recommendations and findings of the present study, consumption of fish low in methylmercury but rich in essential n-3 LCPUFA could be recommended to population.

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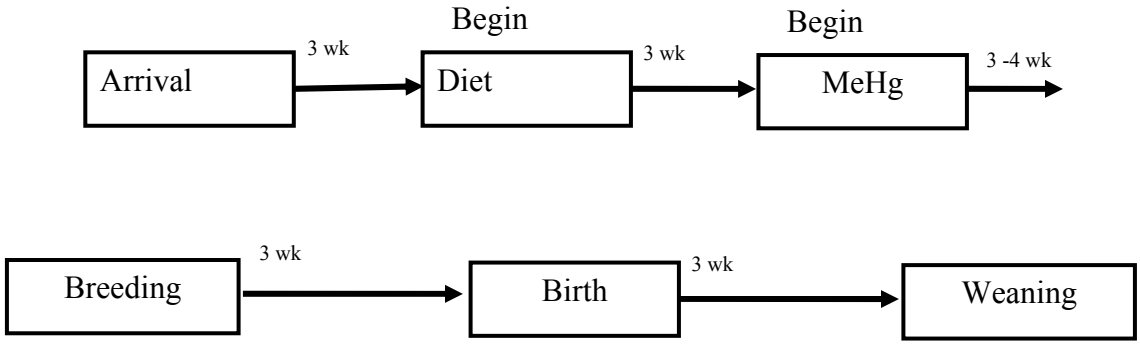
APPENDICES

Appendix A: Experimental timeline



Appendix B: Lipid extraction from cardiac tissue, thin layer chromatography, and methylation of fatty acids

Lipid extraction from cardiac tissue

1. Label four large size (25 x 150 mm) tubes, four medium (16 x 125 mm) and 6 small (13 x 100 mm) tubes with lined Teflon caps. Put Teflon tape on the medium sized tubes.
2. Take tissue sample out of freezer and place tube in beaker with room temperature water.
3. Weight out 0.3 g tissue and add distilled water at a concentration of 0.15 g/1 mL water (e.g. if 0.3 gram sample, use 2.0 mL of distilled water).
4. Homogenize for 5 minutes, or until homogeneous using Omni-homogenizer.
5. Put 1mL of homogenate in large test tube and add 20 mL solvent (chloroform: methanol = 2:1; methanol containing 50 mg BHT/L).
6. Flush the tube with nitrogen, screw on cap tightly and vortex for 15 minutes.
7. Filter the homogenate using Whatman #4 filter paper into another large tube. Rinse original tube with 2 mL chloroform: methanol (2:1) solvent and pour through filter paper.
8. Add 4.6 mL of 0.58% NaCl solution to the test tube and add 1mL of lower phase solvent (chloroform: methanol: water 86:14:1) to the tube.

9. Flush the tube with nitrogen and cap tube; vortex for 5 minutes.
10. Centrifuge the test tubes at 5°C for 10 minutes at 2000 rpm.
11. Use a disposable pipette to remove the supernatant (upper phase), and add 2 mL of upper phase (chloroform: methanol: water 3:48:47).
12. Vortex for 2 minutes, centrifuge at 5° C for 5 minutes at 2000 rpm. Evaporate the lower layer under nitrogen until an oil-like appearance occurs.
13. Add 1 mL dichloromethane (DCM) and vortex for 5 seconds. Transfer the dissolved sample to small size test tube. The small tube was weighed before transferring the solution.
14. Evaporate again and weigh the samples.
15. Reconstitute the sample using 1mg lipid/100 μL DCM.

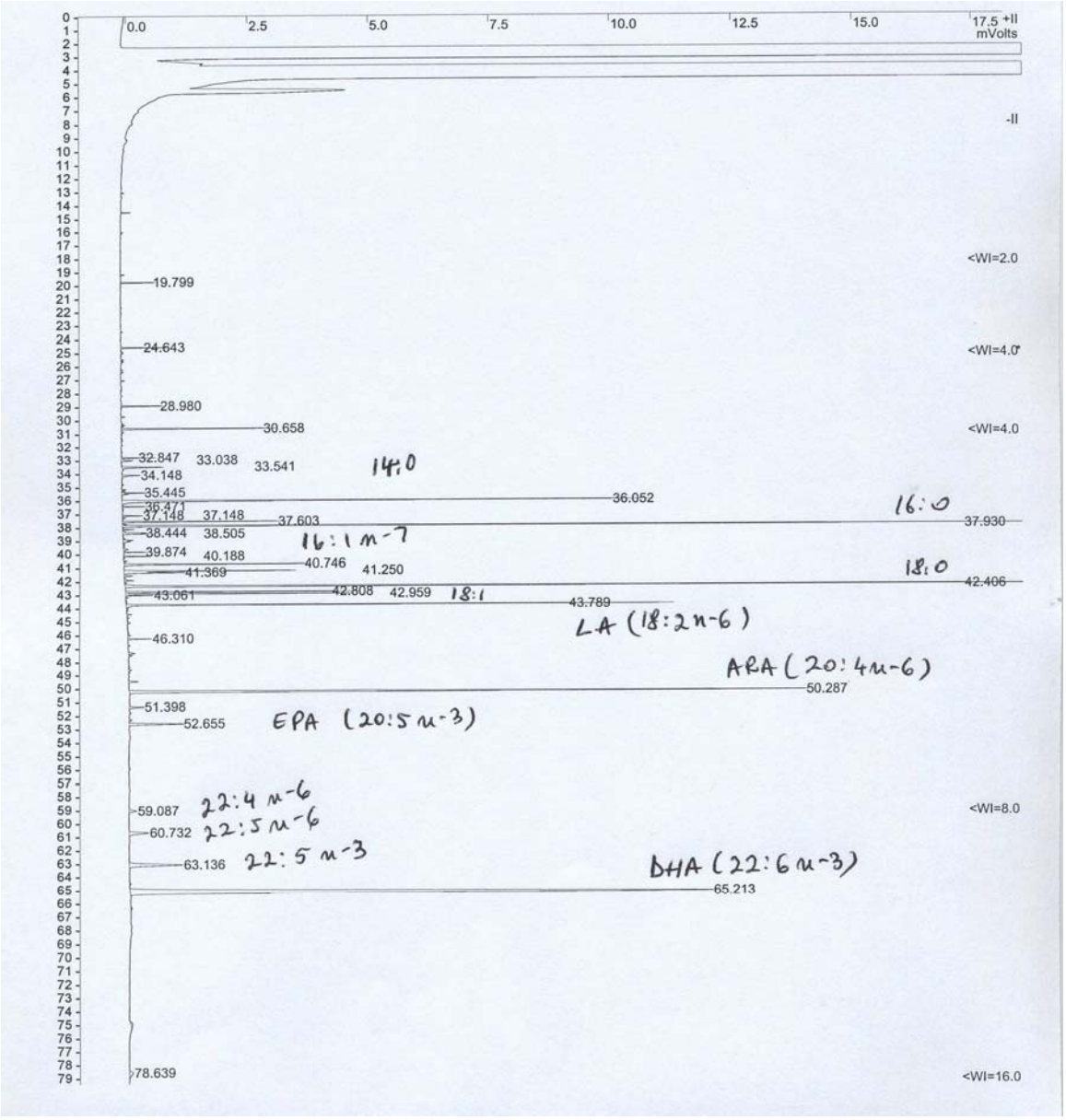
Thin layer chromatography (membrane phospholipids separation)

1. Bake 20 x 20 cm, 250 Microns Silica gel-G # 01011 TLC plate at 120°C (Analtech Inc., Newark, DE) for at least 1 hour.
2. Prepare solvent tank using a chloroform: methanol: acetic acid: water (50:25:6:1.5) add a piece of chromatography paper to tank and seal with tape. Let tank equilibrate for 2-3 hours.
3. Carefully remove silica gel TLC plate from oven and cut in half with glasscutter.
4. Draw a line down the center of the plate. Spot half of the TLC plate with 200µL of extracted sample 1 cm from bottom of the plate. Spot PE and PC standards on the other half. Put the plate into the tank. Allow the solvent front to migrate to within approximately 1.5 cm of the top of the plate.
5. Remove the plate from the tank and let dry. Draw a line approximately 0.5 inches into the sample half of the plate. Cut the plate along this line.
6. Spray the standard/sample portion of the plate with a 50% H2S04 solution. Put the sprayed plate into the oven until the phospholipid bands can be visualized.
7. Scrape the PC and PE bands of the unsprayed portion of the TLC plate into the labeled test tubes (lined with Teflon tape).

Methylation of fatty acids

1. Add 1.0 mL of 10% boron trifluoride solution into each tube. Flush with nitrogen and seal tightly.
2. Place tubes on the heating block at 110°C for 30 minutes. Check the tubes for leakage after 5 minutes.
3. Remove the tube form the heating block and cool immediately for 5-10 minutes.
4. Add 1.0 mL distilled water and 2.0 mL pentane and flush the tube with nitrogen.
5. Vortex for 2 minutes and centrifuge at 5° C for 5 minutes at 2000 rpm.
6. Transfer the supernatant to small tube and evaporate under nitrogen.
7. Add 80 μL of DCM and transfer to a 1 mL GC vial.

Appendix C: Gas chromatogram



Conditions: GC Varian 3900
Autosampler 8410
Column: Omegawax, 250, 30 m x 250 mm I.D., 0.25 µm
Oven: continuous gradient 50-220 ^{0}C at 4^{0}C/ min
followed by a hold time of 35min
Injector: type 1177, 250 ^{0}C
Detector: type FID, splitless, 260 ^{0}C
Carrier gas: helium, 1.0 ml/min
Sample: 1 µl of FAME dissolved in DCM

Appendix D: Protein oxidation detection and Western Blot

Protein Oxidation Detection (OxyBlot procedure)

1. PROTEIN EXTRACTION

Homogenization of tissue: 35-40 mg of tissue is homogenized in 1mL lysis buffer for 60 sec in 2 mL disposable tubes. Transfer the homogenate to 2 ml ependorf tubes.

Centrifugation of homogenate at 4^0 C, 12000g for 10 min. Transfer supernatant in 1.5 mL ependorf tubes. Store protein lysate at -80^0 C.

Lysis buffer preparation: add 1 tablet of protease inhibitors cocktail per 10 mL lysis buffer before homogenization and store the remainder at -20^0 C.

Protein concentration assay (Bio-Rad DC Protein Assay): Lowry assay performed in triplicate.

Tube	lysis buffer (µL)	BSA (µL)
0	100	0
5	95	5
10	90	10
20	80	20
40	60	40
80	20	80
Tube	lysis buffer (µL)	Sample (µL)
5	95	5

Perform BSA standard curve and determine protein concentration using equation.

2. DERIVATIZATION OF CARBONYL GROUPS

The carbonyl groups of proteins are derivatized to 2,4-dinitrophenylhydrazone (DNPH).

15-20 μg of protein is recommended per derivatization reaction.

Denature 5 μL aliquot of protein with 5 μL of 12% SDS for a final concentration of 6% SDS.

Derivatize by adding 10 μL of 1X DNPH solution.

Incubate at room temperature for 15 min.

Add 7.5 μL of Neutralization solution. Add 1.5 μL 2-mercaptoethanol.

When a sample volume other than 5 μL is used must adjust the volume of the other reagents accordingly. Derivatized samples can be stored at 4^0C for up to one week.

3. GEL ELECTROPHORESIS (SDS-PAGE)

Load protein standard, derivatized samples, and OxyBlot standard on gel.

Small gel with 10 wells (BioRad) - run at 40V initially, and then at 80V.

Large gel with 15 wells (made fresh) - run in cold room at constant 25 mA.

4. WESTERN BLOT

Transfer to a membrane: use PVDF membrane (BioRad)

Semi-dry transfer (BioRad) for 1 hr at 10V.

Wet transfer (BioRad) overnight at 30V.

Transfer buffer: 25mM Tris, 192 mM glycine, 20% methanol, pH 8.3

(3.03 g Tris base + 14.4 g glycine + 200 mL methanol + adjust volume to 1 liter with water)

Block non-specific sites

Incubate membrane in blocking buffer for 1hr at room temperature with gentle shaking.

Blocking buffer: 1%BSA/PBS-T (add 5 g BSA to 500 mL PBS-T solution)

Incubate in primary antibody

Dilute 1^0 Antibody 1:150 in Blocking buffer before use. (133.3 µL in 20 mL buffer)

Incubate membrane in 1^0 Antibody solution for 1hr at room temperature with gentle shaking.

Rinse membrane 2 times with 1X PBS-T. Wash once for 15 min and twice for 5 min each at room temperature.

Washing buffer: 1X PBS-T = PBS, pH 7.2-7.5, (Mediatech Inc., Herndon, VA) containing 0.05% Tween 20 (Add 250 µL Tween 20 to 500 mL PBS)

Incubate in secondary antibody:

Dilute 1^0 Antibody 1:300 in Blocking buffer before use. (133.3 µL in 20 mL buffer)

Incubate membrane in 2^0 Antibody solution for 1hr at room temperature with gentle shaking.

Rinse membrane 2 times with 1X PBS-T. Wash once for 15 min and twice for 5 min each at room temperature.

Add Chemiluminescent reagent: use ECL Plus (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK)

Mix 1 mL solution A (big bottle) + 25 µL solution B (small bottle). Cover with foil and allow sitting for 5 min.

Expose for 60-120 sec.

Appendix E: RNA isolation from animal and fibrous tissues

RNA isolation from animal tissue

(RNeasy Mini Kit, Qiagen Inc.)

1) Add β -ME per 1 ml Buffer RLT.
2) Weight out the appropriate amount of fibrous tissue using analytical scale (≤ 30 mg tissue). Do not allow tissue to thaw before placing in Buffer RLT.
3) Place the weighed tissue in a suitably sized vessel for homogenization. Add 1 mL Buffer RLT, and homogenize immediately (60sec) until the sample is completely disrupted.
4) Centrifuge the tissue lysate for 3 min at 10,000 rpm at room temperature. (a small pellet and a layer of film may be present).
5) Carefully transfer the supernatant into a new 1.5 ml or eppendorf tube. (Avoid transferring any of the pellet or film!).
6) Add 1 volume of 70 % ethanol to the cleared lysate. Mix well by pipetting.
7) Pipet 700 µl of the sample into an RNeasy Mini Spin Column in a 2ml collection tube. Close tube gently and centrifuge for 15 sec at 12,000 rpm. Discard the flow-through (re-use the collection tube).
8) Repeat step 7 using the remainder of the sample. Discard the flow-through (re-use the collection tube).
9) Pipet 350 µl Buffer RW1 into the RNeasy Spin Column, and centrifuge for 15 sec at 12,000 rpm to wash. Discard flow-through (re-use the collection tube).
10) Add 10 µl DNase I stock solution (aliquots in -20 freezer) to 70 µl Buffer RDD. Mix by gently inverting the tube. Do not vortex. Pipet the DNase I stock solution

(80 µl) directly on top of the RNeasy silica-gel membrane, and leave it at room temperature for 15 min.

11) Pipet 350 µl Buffer RW1 into the RNeasy Spin Column, and centrifuge for 15 sec at 12,000 rpm. Discard flow-through and collection tube.

12) Transfer the RNeasy Spin Column into a new 2ml collection tube. Pipet 500 µl Buffer RPE onto the RNeasy Spin Column. Close tube gently.

13) Centrifuge for 15 sec at 12,000 rpm to wash the column. Discard flow-through (re-use collection tube).

14) Add another 500 µl Buffer RPE onto the RNeasy Spin Column. Centrifuge for 2 min at 12,000 rpm to dry the membrane.

15) Place the RNeasy Mini Spin Column into a new 1.5 ml collection tube (with cap cut off). Pipet 30 µl RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently and centrifuge for 1 min at 12,000 rpm to elute.

16) Repeat this elution step using the eluate just generated in step15. This will increase your total RNA concentration.

17) To determine the concentration of the RNA:

Make a 1:50 dilution of the RNA in TE Buffer (2 µl RNA + 98 µl TE Buffer, pH 7.5 into disposable BioRad cuvette). TE buffer will be used as blank. Read the absorbance at 260 nm and 280 nm in the spectrophotometer. RNA purity is assessed by calculating the A_{260}/ A_{280} ratio.

RNA isolation from fibrous tissue

(RNeasy Fibrous Tissue RNA Isolation Protocol, Qiagen Inc.)

1. Heat a water bath or heating block to 55° C for proteinase K digestion in step 5.
2. Weight out the appropriate amount of fibrous tissue using analytical scale (≤ 30 mg tissue).
3. Place the weighed tissue in a suitably sized vessel for homogenization. Add 300 µl Buffer RLT, and homogenize immediately (60 sec).
4. Add 590 µl RNase-free water to the homogenate. Then, add 10 µl proteinase K solution and mix thoroughly by pipetting.
5. Incubate at 55° C for 10 min.
6. Centrifuge for 3 min at 10,000 rpm at room temperature. (a small pellet and a layer of film may be present).
7. Pipet the supernatant (~ 900 µl) into a new 1.5 ml or eppendorf tube. (Avoid transferring any of the pellet or film!).
8. Add 0.5 volumes (usually 450 µl) of ethanol (96-100%) to the cleared lysate. Mix well by pipetting.
9. Pipet 700 µl of the sample into an RNeasy Mini Spin Column in a 2 ml collection tube. Centrifuge for 15 sec at ≥ 8000 rpm. Discard the flow-through (re-use the collection tube).
10. Repeat step 9, using the remainder of the sample. Discard the flow-through (re-use the collection tube).
11. Pipet 350 µl Buffer RW1 into the RNeasy Spin Column, and centrifuge for 15sec at ≥ 8000 rpm to wash. Discard flow-through (re-use the collection tube).

12. Add 10 µl DNase I stock solution (aliquots in -20 freezer) to 70 µl Buffer RDD. Mix by gently inverting the tube. Do not vortex. Pipet the DNase I stock solution (80 µl) directly on top of the RNeasy silica-gel membrane, and leave it at room temperature for 15min.
13. Pipet 350 µl Buffer RW1 into the RNeasy Spin Column, and centrifuge for 15 sec at ≥8000 rpm. Discard flow-through and collection tube.
14. Transfer the RNeasy Spin Column into a new 2ml collection tube. Pipet 500 µl Buffer RPE onto the RNeasy Spin Column.
15. Centrifuge for 15 sec at ≥ 8000 rpm to wash the column. Discard flow-through (re-use collection tube).
16. Add another 500 µl Buffer RPE onto the RNeasy Spin Column. Centrifuge for 2 min at ≥ 8000 rpm to dry the membrane.
17. To elute, transfer the RNeasy Mini Spin Column to a new 1.5 ml collection tube. Pipet 30 µl RNase-free water directly onto the RNeasy silica-gel membrane. Centrifuge for 1 min at ≥ 8000 rpm to elute.
18. Repeat this elution step using the elute just generated in step17.
19. To determine the concentration of the RNA:

Make a 1:25 dilution of the RNA in TE Buffer (2 µl RNA + 48 µl TE Buffer → disposable BioRad cuvette).Read the absorbance at 260 nm and 280 nm in the spectrophotometer.

NOTES:

- This kit is to be used for total RNA isolation from fibrous tissues such as heart, aorta, and skeletal muscle.

- A *MAXIMUM* amount of 30 mg of tissue should be used for this procedure in order to obtain optimal yield and purity.
- If first time using kit: β-ME must be added to Buffer RLT before use (10 µl β -ME per 1ml Buffer RLT)
- Add 4 volumes of ethanol (96-100 %) to the Buffer RPE
- All steps should be carried as quickly as possible, and at room temperature, unless otherwise indicated.

Appendix F: cDNA synthesis and RT-PCR method

cDNA synthesis

(iScript cDNA synthesis kit, Bio-Rad)

REAGENTS

1. 5x iScript Reaction Mix
2. Nuclease-free water
3. iScript Reverse Transcriptase

REACTION SET-UP

Component	Volume per reaction
5x iScript Reaction Mix	4 µl
iScript Reverse Transcriptase	1 µl
Nuclease-free water	x µl
RNA template (1 µg total RNA)	x µl
Total volume	20 µl

REACTION PROTOCOL

Incubate complete reaction mix:

5 min at 25° C

30 min at 42° C

5 min at 85° C

Hold at 4° C (optional)

RT-PCR

(iQ SYBR Green Supermix, Bio-Rad)

MASTER MIX SET-UP: 1 tube/animal/primer

Component	Volume per reaction
Sterile water	9 µl
Primer mix	3 µl
cDNA	0.5 µl
SYBR Green	12.5 µl
Total volume	25 µl

Add 25 µl of master mix to each well on the plate according to the plate layout. Perform the procedure in triplicate for both, the housekeeping gene and gene of interest.

Appendix G: Chemical reactions of the oxidative system enzymes

NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase): generates superoxide by transferring electrons to molecular oxygen to produce the superoxide

$$NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^- + H^+$$

Superoxide dismutase (SOD): catalyses dismutation of superoxide:

$$O_2\cdot^- + O_2\cdot^- + 2\ H+ \rightarrow O_2 + H_2O_2$$

Glutathione peroxidases (GPX): reduction of peroxides coupled to oxidation of glutathione:

$$2\ GSH + H_2O_2 \rightarrow GS\text{-}SG + 2\ H_2O$$

Catalase (CAT): dismutation of hydrogen peroxide

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Appendix H: Calculation on essential fatty acid equivalence

Assumption: n-3 LCPUFA diet fed to the animals is comparable to human requiremnts.

Calculations were performed using Metabolic Body weight = $(\text{Body wt})^{0.75}$

Calculation for adult requirements:

Adult 70 kg, recommended 200 mg – 4000 mg LCPUFA/ day

$(70 \text{ kg})^{0.75}$ = 24.2 kg Metabolic BW

200 mg LCPUFA/ 24.2 kg Metabolic BW = 8.26 mg/kg/day n-3 LCPUFA

2000 mg LCPUFA/ 24.2 kg Metabolic BW = 82.6 mg/kg/day n-3 LCPUFA

4000 mg LCPUFA/ 24.2 kg Metabolic BW = 165.3 mg/kg/day n-3 LCPUFA

Calculation for animals:

Animal BW: 300 g, eating about 20 g food/ day

Food contained 4 % fat with 10 % n-3 LCPUFA

20 g food of 4 % fat: (20x4)/100 = 0.8 g fat

0.8 g fat of 10 % LCPUFA = 0.08 g LCPUFA = 80 mg n-3 LCPUFA

$(0.3 \text{ kg})^{0.75}$ = 0.405 kg Metabolic BW rat

80 mg n-3 LCPUFA/ 0.405 kg Metabolic BW = 197.5 mg/kg/day n-3 LCPUFA

Answer: The n-3 LCPUFA content of the diet fed to animals was within physiological limits at the high normal of recommendation for human.

Appendix I: Mercury content of heart tissue

Total Hg content (μg/g) in the heart

(-) n-3LCPUFA			(+) n-3LCPUFA		
0 MeHg	0.5 MeHg	5 MeHg	0 MeHg	0.5 MeHg	5 MeHg
$< 0.05 \pm 0.0^{a}$	0.081 ± 0.02^{a}	12.72 ± 1.7^{b}	$< 0.05 \pm 0.0^{a}$	0.068 ± 0.02^{a}	10.68 ± 0.7^{b}

Animals were exposed for 12 months to 0, 0.5, and 5 ppm in drinking water and fed a diet with (+) or without (-) n-3 LCPUFA.
Means within a row with different superscript letters are significantly different by two-way ANOVA with post hoc Tukey-Kramer adjustment. A significant Hg effect was observed ($P < 0.0001$).