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KARI SEPPÄNEN

Does mercury promote lipid peroxidation?

In *vivo* and in *vitro* studies concerning mercury and selenium in lipid peroxidation and coronary heart disease

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L22, Snellmania building, University of Kuopio, on Friday 24th September 2004, at 12 noon

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Department of Applied Physics

Author's address:
Department of Chemistry
University of Kuopio
P.O. Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 163 251
Fax +358 17 163 259
E-mail: kariilmari.seppanen@uku.fi

Supervisors:
Professor Jukka T. Salonen, M.D., Ph.D., M.Sc.P.H.
Research Institute of Public Health
University of Kuopio

Professor Reino Laatikainen, Ph.D.
Department of Chemistry
University of Kuopio

Supervisor assistant Simo Lötjönen, Ph.D.
Department of Chemistry
University of Kuopio

Reviewers:
Professor (emeritus) Pentti Kauranen, Ph.D.
Department of Chemistry
University of Kuopio

Professor Jyrki Liesivuori, Ph.D.
Department of Pharmacology and Toxicology
University of Kuopio

Opponent:
Docent Georg Alfthan, Ph.D.
Department of Health and Functional Capacity
National Public Health Institute (KTL)
Helsinki

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ABSTRACT

The aim of this study was to examine *in vivo* and *in vitro* the mechanism of action of mercury in lipid peroxidation and to investigate if it is possible to reduce on the potential damage induced via this mechanism.

The starting point for these studies was the observed significant correlation between a high intake of mercury, apparently from consumption of freshwater predatory fish, and the consequent accumulation of mercury in the body. This accumulation was associated with an excess risk of acute myocardial infarction as well as death from coronary heart disease and cardiovascular disease in eastern Finnish men. It was postulated that this increased risk was attributable to the promotion of lipid peroxidation by mercury.

The effects of mercury, copper and iron on the reactions catalyzed by the enzymes glutathione peroxidase and myeloperoxidase were investigated *in vitro.* Direct effects of mercury on peroxidation of LDL were studied by using proton nuclear magnetic resonance (^{1}H NMR). According to NMR results, mercury, alone or in combination with hydrogen peroxide, does not catalyze the peroxidation of LDL. The results of the enzyme studies indicate that mercuric ion is a strong inhibitor of glutathione peroxidase but in the presence of LDL it has no effect on myeloperoxidase. Cupric ion is a powerful activator of myeloperoxidase (MPO) and simultaneously, it a strong enhancer of the inhibitory effect of mercury on glutathione peroxidase (GSH-Px) and, thus, it completely prevents the protective effect of GSH-Px on the peroxidation of LDL. *In vitro,* ferric ion had no effect on the activity of glutathione peroxidase but it did enhance the activity of MPO.

Selenium compounds can interact readily with mercury making it chemically inactive. Selenium is in fact the only potential element which could be expected to regulate mercury levels in living systems though the effects of two selenium forms, inorganic and organic selenium, would be anticipated to have different time scales of effects in these systems. *In vivo*, the mercury-binding capacity of organic and inorganic selenium was assessed using rats as experimental animals with the effects of selenium on human mercury status being assessed by supplementation with organic selenium. According to these studies, both inorganic and organic selenium can lower mercury levels in animals and also in humans. Even a small amount of inorganic selenium in food seems to reduce the absorption of mercury into the blood but organic selenium was ineffective, although this form was also found to lower the mercury level in the human body. The results support the hypothesis that selenium has a protective effect against mercury. Thus, the same mechanism involved in the inacvation of the glutathione peroxidase enzyme, the high affinity of mercury to selenium, can be beneficial in the opposite direction.

In this work, a molecular level model for the action of mercury on lipid peroxidation has been proposed. This model accounts for the role of mercury on LDL peroxidation and thus offers an explanation for the previous epidemiological and *in vivo* observations. It can be concluded that mercury does not promote lipid peroxidation directly via radical production, and in that respect it differs from copper and iron, instead it acts via the glutathione peroxidase enzyme. Thus, also *in vivo* high levels of copper and iron when combined with a high exposure of mercury and a low intake of selenium confer an excess risk of coronary heart disease.

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To my mother

"...only fishing is important..."
Unknown

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Kuopio, August 2004 Kari Seppänen

ABBREVIATIONS

AMI	acute myocardial infarction
$CDCl_3$	deuterochloroform
CHD	coronary heart disease
CH_3Hg^+	methyl mercury
CVD	cardiovascular disease
DMPS	2,3-dimercaptopropanesulfonic acid sodium salt monohydrate
DPPD	N,N′-diphenyl-p-phenylenediamine
GSH	glutathione
GSH-Px	glutathione peroxidase
GSSG	glutathione disulphide
HDL	high density lipoprotein
$HgCl_2$	mercuric chloride
^1H NMR	proton nuclear magnetic resonance
H_2O_2	hydrogen peroxide
HOC	hypochlorous acid
LDL	low density lipoprotein
MPO	myeloperoxidase
NAD	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
$O_2^{\cdot -}$	superoxide anion
OH^-	hydroxyl ion
$^{\cdot}OH$	hydroxyl radical
SH	sulfhydryl

LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following original publications referred to in the text by their Roman numerals **I-IV**:

I. Salonen J. T., Seppänen K., Nyyssönen K., Korpela H., Kauhanen J., Kantola M., Tuomilehto J., Esterbauer H., Tatzber F., Salonen R. Intake of Mercury From Fish, Lipid Peroxidation, and the Risk of Myocardial Infarction and Coronary, Cardiovascular, and Any Death in Eastern Finnish Men. *Circulation* **1995**, 91, 645-655.

II. Seppänen K., Laatikainen R., Salonen J. T., Kantola M., Lötjönen S., Harri M., Nurminen L., Kaikkonen J., Nyyssönen K. Mercury-Binding Capacity of Organic and Inorganic Selenium in Rat Blood and Liver. *Biol. Trace Elem. Res.* **1998,** 65, 197-210.

III. Seppänen K., Kantola M., Laatikainen R., Nyyssönen K., Valkonen V. P., Kaarlöpp V., Salonen J. T. Effect of Supplementation with Organic Selenium on Mercury Status as Measured by Mercury in Pubic Hair. *J. Trace Elements Med. Biol.* **2000,** 14, 84-87.

IV. Seppänen K., Soininen P., Salonen J. T., Lötjönen S., Laatikainen R. Does Mercury Promote Lipid Peroxidation? An *In Vitro* Study Concerning Mercury, Copper and Iron in Peroxidation of Low Density Lipoprotein. *Biol. Trace Elem. Res.* **2004**, accepted for publication.

CONTENTS

1. INTRODUCTION

Mercury belongs to group IIB in the periodic system. Although it has a full d-shell, it has properties which often include it in the transition metals. The human body contains about 13 mg of mercury[1], but so far as is known it has no biological function. Many centuries ago, mercury was used medically to cure syphilis but nowadays it has no medical use and it is classified as a highly neurotoxic element. Toxicologists have been very interested in mercury because in adition to damaging the kidney and liver, it can also evoke neurological disturbances, for example deficits in neurocognitive function such attention, fine-motor function and verbal memory.[2] Also the immunotoxic effect of mercury has been examined in some studies.[3]

In the aqueous solutions mercury can exist in two redox states, as a mercurous ion Hg_2^{2+} or as a mercuric ion Hg^{2+}. Both of these states are stable in physiological conditions and, in principle, they form a similar pair as the Cu^{+}/Cu^{2+} and Fe^{2+}/Fe^{3+}-pairs which are involved in numerous biological events. Therefore it could be expected, for example, that the Hg_2^{2+}/Hg^{2+}-pair could have a role in biological (per)oxidation systems. On the other hand, there are properties of mercury that make it different from those other valency pairs and from any other element in the periodic system. The mercury ions are very soft and therefore they have a high affinity for sulfur. This together with the unique property to form non-ionic (also in water) lipid soluble chloride $HgCl_2$, methylmercurychloride MeHgCl and methylmercury $Hg(Me)_2$ explain its pronounced neurotoxicity: the mercury compounds accumulate into neuron cell membranes and react with ATP-ase causing permanent damage.[4] A further unique property of mercury is its high affinity for selenium. These properties, together with epidemiological and biological evidence, make mercury a potential causative factor in the etiology of atherosclerosis.

The concentration of mercury is highest in the lakes of eastern Finland because of the acidity of these lake waters.[5] This increases the availability of mercury and its accumulation into fish.[6] Fish and fish products are the major sources of methyl mercury in food. Methyl mercury is rapidly accumulated by most aquatic biota and it attains its highest concentration in predatory fishes, such as the northern pike, at the top of the aquatic food chain.

A high fish intake has been associated with a reduced mortality from coronary heart disease (CHD) in several prospective population studies.[7-10] However, in at least three studies in populations with high fish intakes, no such relation was observed.[11-13] Also, even though men in

eastern Finland consume a lot of fish,[14] their mortality from CHD is one of the highest in the world.[15] This is at odds with the concept that a high intake of fish would uniformly be healthy for the cardiovascular system. Since in some populations[11-13] a high intake of fish did not appear to be associated with reduced CHD mortality, it could be argued that either differences have to exist in the nutrient composition of the fish, or possibly some harmful substances in fish might account for these inconsistencies. In support of this latter possibility it is possible that mercury in fish could counteract the beneficial metabolic effects of other nutrients present in fish.

Previously, a relationship has been observed between selenium deficiency and an excess risk of acute myocardial infarction (AMI) as well as death from CHD and cardiovascular disease (CVD) in eastern Finland.[16] The finding has also been confirmed in another prospective population study[17] and in a case-control study.[18] A relationship has also been found between low serum selenium levels[19] and lipid peroxidation in vivo[20] with accelerated progression of carotid atherosclerosis in eastern Finnish men. It is believed that lipid peroxidation may have an important role in the etiology of many pathological conditions including atherosclerosis.[20,21] Divalent metal ions of iron and copper are known to promote lipid peroxidation[22] and also, both mercuric and methyl mercuric chlorides can induce lipid peroxidation in rats.[23,24]

Peroxidation of blood lipoproteins is regarded as a key event in the development of atherosclerosis.[25] Peroxidation of LDL (low density lipoprotein) in the intima of large arteries is initiated by free radicals, which are likely formed in the course of inflammatory processes that are mediated by enzymes such as lipoxygenase and myeloperoxidase.[26-30] Once initiated by a generator of free radicals, peroxidation is further catalyzed by free or chelated transition metal ions, particularly iron and copper.[25] *In vitro*, iron ions promote LDL peroxidation only in the presence of free radical generators[31] whereas copper ions, at micromolar concentrations, can form free radicals at the LDL surface[32-34] by interacting with LDL associated antioxidants or with pre-formed, LDL associated hydroperoxides.[25]

There is strong epidemiological evidence for an association between a high body mercury concentration and accelerated atherosclerosis leading to an excess risk of acute myocardial infarction and death from coronary heart disease.[35-37] However, the role of mercury in these processes is unclear. It has been proposed that mercury promotes lipid peroxidation in the same way as copper and iron.[35] Although the Hg_2^{2+}/Hg^{2+} redox-pair makes the mechanism plausible, from the chemical point of view this explanation is not convincing because the redox and

coordination properties of mercury differ greatly from those of copper and iron. On the other hand, the high affinity of selenium for mercury and the essential role of selenium in the active site of glutathione peroxidase[38] suggest that the interactions of and the balance between mercury, copper, iron and selenium in the human body could well affect lipid peroxidation. Selenium is known to have an important role in the regulation of the toxic effects of mercury.[39]

In order to explain the association between mercury, atherosclerosis and coronary heart disease, the mechanism of action of mercury in lipid peroxidation needs to be clarified. Although the roles of copper and iron have been widely studied less is known about mercury. It has been proposed that mercury ions and mercurial compounds can inhibit SH-dependent enzymes and metabolites, NADP- and NAD-dependent metabolic reactions, and promote oxidative stress by enhancing the supply of hydrogen peroxide or by blocking the capture of radicals,[40] as well as via undefined mechanisms. For instance, the effects and interactions of mercury, copper and iron in the enzymatic peroxidation of LDL have not been fully clarified.

2. AIMS AND IMPLEMENTATION OF THE PRESENT STUDY

The aims of this study were:

1. To investigate the possible association between mercury, lipid peroxidation and coronary heart disease
2. To determine the mechanism of action of mercury in lipid peroxidation
3. To clarify interactions between mercury and selenium in lipid peroxidation
4. To investigate how the action of mercury in lipid peroxidation can be inhibited by selenium

The implementation of the present study is based on the separate original publications (**I-IV**). The methods and experiments in detail are described in these publications. The possible association between mercury content of the human body and lipid peroxidation and coronary heart disease was discovered in original publication **I**. The effects of selenium on animal and human mercury status have been followed up in publications **II** and **III**. The mechanism of action of mercury in peroxidation of low density lipoprotein has been explored in publication **IV**.

3. ASSOCIATION OF MERCURY WITH LIPID PEROXIDATION AND CORONARY HEART DISEASE

3.1 Mercury sources in Finnish food

The principal sources of mercury in Finnish food are especially freshwater predatory fishes especially burbot (*Lota lota*), northern pike (*Esox lucius*) and perch (*Perca fluviatilis*),[35,41,42] in contrast the mercury contents of other Finnish foodstuffs are quite low. In the years 1982-1993, mercury concentrations of 0.24-0.88 μg/g of tissue were measured in northern pikes of Finnish natural lakes with 96% of the mercury being in the form of methyl mercury.[43] In a study which surveyed the development of mercury concentrations in fish in reservoirs and lakes situated in western and northern Finland over a period of 16 years (1979-1994), the mean Hg concentrations in standardized 1 kg pike from 1989 to 1993 was 0.45 mg/kg in natural lakes and 0.58 mg/kg in reservoirs.[41] In contrast to western Finland, for example in a lake in eastern Finland, Koitere, the mercury concentrations of all studied fish species were high in the years 2000-2001.[42] Northern pike had mercury concentration of 1.0 mg/kg already when their weight was less than 2 kg and the mercury concentrations of all examined perches and burbots were over 0.5 mg/kg.

3.2 Mercury and coronary heart disease (I)

The role of lipid peroxidation in atherogenesis has been confirmed in both basic research[44-46] and epidemiologic studies.[20,47,48] Lipid peroxidation in the human body is regulated by free radical stress, the availability of pro-oxidative catalytic metals and antioxidative defense systems.[44-46] Mercury, especially methyl mercury, can promote lipid peroxidation.[37] The principal sources of mercury intake are diet, and predatory fish are the main dietary source of mercury in most industrialized countries. In fish, mercury is present in an organic form, mainly as methyl mercury. It is well absorbed from the gut and accumulates in epithelial tissues including hair. The mercury content of hair can be used as an indicator of mercury intake over several months.[35]

Even though previous studies[7-10] have pointed to an association between high fish intake and reduced coronary heart disease (CHD) mortality, men in eastern Finland who have a high fish intake, have an exceptionally high CHD mortality. It can be hypothesized that this paradox could in part be explained by the high mercury content in the fish consumed in eastern Finland.

The relationship of the dietary intake of fish and mercury, as well as hair mean content and urinary excretion of mercury, to the risk of acute myocardial infarction (AMI) and death from CHD, cardiovascular disease (CVD), and any cause was studied in an epidemiologic study in 1833 men aged 42 to 60 years who were free of clinical CHD, stroke, claudication, and cancer. Of these, 73 experienced an AMI between 2 to 7 years after this survey. Of the 78 deceased men, 18 died of CHD and 24 died of CVD. Men who had consumed local non-fatty fish species had elevated hair mercury contents (Table 1). In Cox models with the major cardiovascular risk factors as covariates, dietary intakes of fish and mercury were associated with significantly increased risk of AMI and death from CHD, CVD, and any death. Men in the highest tertile (2.0 μg/g) of hair mercury content had a 2.0-fold (95% confidence interval, 1.2 to 3.1; *P*=.005) age- and CHD-adjusted risk of AMI and a 2.9-fold (95% CI, 1.2 to 6.6; *P*=.014) adjusted risk of cardiovascular death compared with those with a lower hair mercury content. In a nested case-control subsample, the 24-hour urinary mercury excretion had a significant (*P*=.042) independent association with the risk of AMI.

In a subsample of the subjects (n=187) for whom the measurements of serum immune complexes containing oxidized LDL existed, both the hair mercury content and the urinary mercury excretion associated with immune complex titers measured with a neat rabbit antiserum against oxidized LDL and the γ-globulin fraction of a rabbit antiserum against oxidized LDL. Both the hair and urinary mercury associated significantly with titers of immune complexes containing oxidized LDL.

The results of this study suggest that a high intake of mercury from freshwater predatory fish and the consequent accumulation of mercury in the body are associated with an excess risk of AMI as well as death from CHD and CVD in eastern Finnish men and this increased risk may be due to the promotion of lipid peroxidation by mercury.

Table 1. Distributions of indicators of body mercury status, dietary intakes of fish mercury, other relevant nutrients and coronary risk factors (**I**)

	Mean	SD	Minimum	Maximum
Hair mercury content, μg/g	1.92	1.98	0	15.67
Urinary mercury excretion, μg/d (n=207)	1.18	1.10	0	4.95
Dietary mercury intake, μg/d	7.6	7.7	1.1	95.3
Dietary fish intake, g/d	46.5	55.5	0	619.2
Age, y	52.4	5.3	42.0	61.2
Lifetime smoking, cigarette-y	148	303	0	2880
Systolic blood pressure, mm Hg	134.0	16.4	87.7	213.3
Serum cholesterol, mmol/L	5.86	1.04	2.60	14.40
Serum LDL cholesterol, mmol/L	4.00	0.98	0.68	8.08
Serum apolipoprotein B, g/L	1.02	0.23	0.01	1.86
Serum HDL_2 cholesterol, mmol/L	0.86	0.27	0.07	2.77
Dietary iron intake, mg/d	18.6	5.6	2.5	50.4
Maximal oxygen uptake, $mL \cdot kg^{-1} \cdot min^{-1}$	32.1	7.2	6.4	65.4
Plasma fibrinogen, g/L	2.97	0.53	1.32	6.71

n = 1833

3.3 Mercury and lipid peroxidation (I)

There are at least three proposed mechanisms through which mercury can promote lipid peroxidation. First, mercury is a metal which has transitional properties, and it can thus act as a catalyst in Fenton-type reactions, resulting in the formation of free radicals. The concept that mercury could promote free radical generation was first presented by Ganther[49] based on the observation that vitamin E and the antioxidant DPPD (N,N′-diphenyl-p-phenylenediamine) provided protection against methyl mercury poisoning in rats.[50] In an *in vitro* study, Hg^{2+} ions at micromolar concentrations increased the production of superoxide anions in human neutrophils.[51] In another *in vitro* study, mercuric ions (1 to 6 μmol/L) caused a concentration-dependent increase (up to fivefold) in mitochondrial H_2O_2 production.[52] In addition to its direct catalytic effect, mercury has been found to enhance iron-stimulated lipid peroxidation *in vitro*.[44]

An *in vivo* study revealed a significant concentration-related depolarization of the inner mitochondrial membrane, increased H_2O_2 formation, glutathione depletion, and formation of

thiobarbituric acid reactive substances after the addition of Hg^{2+} to mitochondria isolated from kidneys of untreated rats.[53] Thus, although catalytic Fe^{2+} principally catalyzes the oxidation of H_2O_2 to the more reactive hydroxyl radical,[54] mercury appears to act earlier in the Fenton reaction chain, catalyzing the production of H_2O_2.

Second, mercury has a very high affinity for sulfhydryl groups, which in plasma proteins have been estimated to account for as much as 10% to 50% of the antioxidative capacity of plasma.[55] By binding to sulfhydryl groups, mercury inactivates antioxidative thiolic compounds such as the glutathione.[56] Glutathione has a central role in the regeneration of the tocopheroxyl radical to tocopherol. Mercury poisoning, which is associated with increased lipid peroxidation in the liver and in the kidneys, also results in inactivation of superoxide dismutase and catalase,[57] two important enzymes that destroy H_2O_2. Thus, thiol antidotes such as DMPS and D-penicillamine can chelate mercury and protect against mercury-induced lipid peroxidation.[57]

Third, mercury can form an insoluble compound mercury selenide when it reacts with selenium[58] and thus, it binds selenium in an inactive form that cannot serve as a component for glutathione peroxidase. Before 1987, the dietary intake and blood concentrations of selenium were exceptionally low in Finland.[16, 59, 60] High mercury intake probably has reduced the bioavailability of selenium even further. Ganther and coworkers[61] have demonstrated that selenium protects against methyl mercury toxicity and selenium has been observed to protect against the peroxidative liver injury caused by mercury.[62]

4. EFFECTS OF SELENIUM ON MERCURY IN LIVING SYSTEMS

4.1 Mercury-binding capacity of selenium in rats (II)

Selenium can affect lipid peroxidation through at least two pathways. First, selenium is a component of the glutathione peroxidase enzyme[36] which destroys lipid peroxides in the human body,[63] and second, selenium compounds can interact readily with mercury making it chemically inactive.[64] Selenium is in fact the only potential chemical which would be predicted to regulate the mercury level in living systems but also the effects of two selenium forms, inorganic and organic selenium, would be expected to have quite different properties in these systems.

The mercury-binding capacity of seleno-DL-methionine and selenium dioxide was assessed in male Wistar rats. Thirty-five rats were randomized into seven groups of five animals. One group was a control group to check for any mercury contamination occurring during the experiment and the other groups were experimental groups. The experiment was divided into two phases. The first phase was a "washout" and stabilization phase and it lasted for 6 weeks. The second phase was the actual experimental phase, which lasted for 4 weeks. Between these two phases, all groups fasted for one day. Mercury was supplied as fish loaves made of northern pike or rainbow trout. The total amounts of supplied mercury during the experiment were 189-249 μg in the northern pike groups and 21-35 μg in the rainbow trout groups. The selenium concentration was 3.4 mg/kg fish, about sixfold compared to the equivalent quantity of mercury.

The purpose of this study was to bind fish mercury to selenium to form mercury selenide already in the reducing conditions of the gastrointestinal tract, and, in this way, to inactivate the mercury. The results of this study indicate that it is possible to inactivate mercury, at least to some extent. The findings concerning dose-response suggest that (Tables 2 and 3) an inorganic selenium compound, selenium dioxide, was effective in the inactivation of mercury in the intestinal tract of the rat. The effect of selenium dioxide was seen both in rat blood (25%) and in the liver (24%) for levels of total mercury and also in blood (29%) for methyl mercury in the northern pike group. On the other hand, seleno-DL-methionine seemed to increase the mercury level both in the blood and in the liver. However, the proportion of methyl mercury to total mercury was lowered. Seleno-DL-methionine can transfer mercury into the blood and react with methyl mercury, eliminating a part of methyl mercury. In the rainbow trout group, the mercury-binding capacity of the organic or inorganic selenium could not be found at all, probably due to the very

low concentration of mercury present in rainbow trouts. The rather low mercury-binding capacity of organic or inorganic selenium may be due to its much faster absorption and metabolism in rat than in humans. For example, the biological half-life of methyl mercury in rats is only 18-22 days,[65] whereas it is 76 days in humans.[66] Thus, it is possible that the binding efficiency in humans would be significantly higher. Also, the short experimental time could have had an effect on the mercury-binding capacity of selenium. However, as the results of the present study indicate, inorganic selenium can inactivate mercury in fish, but it is essential to supply the selenium simultaneously with the mercury.

Table 2. Response indices of total and methyl mercury and the differences between them in the pooled blood of the rats (**II**)

Group	Response index[a]		Difference
	Total mercury[b]	Methyl mercury[b]	
Control			
Group A	0.0	0.0	0.0
Northern pike			
Group B[c]	7.2	6.5	0.7
Group D[d]	7.6	6.2	1.4
Group F[e]	5.4	4.6	0.8
Rainbow trout			
Group C[c]	1.5	1.4	0.1
Group E[d]	1.4	1.2	0.2
Group G[e]	1.5	1.3	0.2

[a]Response index = response/dose. Dose signifies the total intake of mercury (μg) during the experiment and response signifies the concentration of mercury (μg/l) in the pooled blood of the rats at the end of mercury and selenium supplementation.
[b]Calculated from means of the groups (n = 5 in all groups) .
[c]NaCl group (3.5 g NaCl/ kg fish).
[d]Seleno-DL-methionine group (3.4 mg Se/kg fish and 3.5 g NaCl/kg fish).
[e]Selenium dioxide group (3.4 mg Se/kg fish and 3.5 g NaCl/kg fish).
Non-parametric Wilcoxon rank-sum test was used in statistical analyses. The differences were considered significant between groups when the p-value was less than 0.05.

Table 3. Response indices of total and methyl mercury and the differences between them in the liver of the rats (**II**)

Group	Response index[a]		Difference
	Total mercury[b]	Methyl mercury[b]	
Control			
Group A	0.0	0.0	0.0
Northern pike			
Group B[c]	2.9	1.6	1.3
Group D[d]	2.8	1.4	1.4
Group F[e]	2.2[f]	1.1	1.1
Rainbow trout			
Group C[c]	0.8	0.2	0.6
Group E[d]	1.1	0.3	0.8
Group G[e]	0.4	0.2	0.2

[a]Response index = response/dose. Dose signifies the total intake of mercury (μg) during the experiment and response signifies the concentration of mercury (μg/kg) in the liver of the rats at the end of mercury and selenium supplementation.
[b]Mean of the group (n = 5) .
[c]NaCl group (3.5 g NaCl/ kg fish).
[d]Seleno-DL-methionine group (3.4 mg Se/kg fish and 3.5 g NaCl/kg fish).
[e]Selenium dioxide group (3.4 mg Se/kg fish and 3.5 g NaCl/kg fish).
[f]$P < 0.01$ vs group B.
Non-parametric Wilcoxon rank-sum test was used in statistical analyses. The differences were considered significant between groups when the p-value was less than 0.05.

4.2 Effect of organic selenium on human mercury status (III)

Of the two elements under study, mercury is a toxic heavy metal, whereas selenium is an essential trace element,[67] but it is also toxic at higher doses with selenium dioxide being much more toxic than seleno-DL-methionine. Selenomethionine in 10 mM solutions evokes only 4% hemolysis whereas selenium dioxide causes 39% hemolysis in rat erythrocytes.[68] The effect of selenium supplementation has been thought to be dependent on the chemical form of selenium, with yeast selenium being more efficient compared to inorganic selenite.[69,70]

The purpose of the present study was to evaluate the effect of four months of yeast-based selenium supplementation on selenium and mercury status in subjects with low serum selenium concentrations. In Estonia the serum selenium levels in the whole population are between 50 and 100 μg/l and the mean value is 70 μg/l, while in Finland the mean serum selenium is near to a level of 100 μg/l.[71] This study was carried out in Rakvere, Estonia.

Pubic hair mercury, serum selenium and blood selenium concentrations in 23 subjects (serum selenium < 90 μg/l) were investigated before and after selenium supplementation. Scalp hair has most often been used as a sample material when the mercury content of human body has been estimated.[35] However, pubic hair is also a suitable way to assess mercury in the human body because its mercury content correlates well with the mercury content of scalp hair. The correlation coefficient between scalp and pubic hair samples was 0.96 in 24 subjects.[72] Ten subjects were randomized into the selenium supplementation group and also ten into the placebo group. The selenium supplementation group received daily 100 μg of selenomethionine as Selena capsules (Novamed, Helsinki, Finland) made of *Saccharomyces cerevisiae*-yeast which contained 82-87% selenomethionine of total selenium. Selenium supplementation (Table 4) reduced pubic hair mercury level by 34% ($p=0.005$) and elevated serum selenium by 73% and blood selenium by 59% in supplemented group ($p<0.001$ for both). As the results of this study indicate, mercury levels in pubic hair could be reduced by dietary supplementation with small daily amounts of organic selenium over a short time of four weeks.

Table 4. Baseline pubic hair mercury, serum selenium and blood selenium concentrations and their changes during the selenium supplementation in the placebo and supplementation groups (III)

Group	Pubic hair mercury[a] µg/kg	Serum selenium[a] µg/l	Blood selenium[a] µg/l
Placebo group (n=10)			
Baseline	469±225	72±12	77±8
End	448±228	73±13	79±7
Change[b]	-21±138	1±4	2±9
Supplementation group (n=10)			
Baseline	415±162	70±13	74±11
End	273±165[c]	121±27[c,e]	118±15[c,e]
Change[b]	142±101[d]	51±22[e]	44±12[e]

[a]Mean ± SD.
[b]Change = End minus baseline.
[c]$p<0.01$ vs baseline.
[d]$p<0.05$ vs placebo group.
[e]$p<0.001$ vs placebo group.
Differences between the groups at baseline and at the end of the selenium supplementation were tested by a non-parametric Wilcoson rank-sum test, and differences between the beginning and the end were tested by a non-parametric Wilcoson matched-pairs test both in the placebo and in the supplementation groups. The differences were considered significant when the p-value was less than 0.05.

5. MECHANISM OF ACTION OF MERCURY IN PEROXIDATION OF LOW DENSITY LIPOPROTEIN (IV)

5.1 Experimental procedure

There are two ways to review lipid peroxidation, it can be divided into the enzymatic and non-enzymatic systems or it can be considered according to the formation or destruction of free radicals in the reactions. A generation and quenching scheme for radicals produced during the peroxidation of LDL is presented in Figure 1. First, to study the enzymatic system, the effects of mercury, copper and iron on glutathione peroxidase (GSH-Px, EC 1.11.1.9) and myeloperoxidase (MPO, EC 1.11.1.7) were investigated by measuring changes in the concentration of the coenzyme NADPH spectrometrically *in vitro* by a Spectronic Genesys-5 UV-VIS spectrophotometer (Spectronic Instruments, Inc., NY, USA). To investigate the effects of the elements on the protection system against peroxides, glutathione peroxidase (Fig. 1, reaction 1), a fixed-time assay was used and the activity of myeloperoxidase (Fig. 1, reaction 2) was investigated by a kinetic assay with measurement points at 0, 5, 10, 15 and 30 minutes (Fig. 2 and 3). Further more, because transition metal ions are known to catalyze the decomposition of peroxides, the direct effects of mercury, copper and iron on hydrogen peroxide and the influence of hypochlorous acid on the myeloperoxidase activity were assessed by measuring the amounts of NADPH consumed (μmol/l · min) both in enzymatic and non-enzymatic reactions. The starting concentration of NADPH in these reactions was 0.25 mmol/l and both of these reaction mixtures were incubated for 15 minutes at +37°C. Second, to explore the effects of mercury, copper and iron on the direct oxidation of LDL and because the measurement of NADPH does not reveal the target of the enzymatic peroxidation (Fig. 1, reaction 3), proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used.

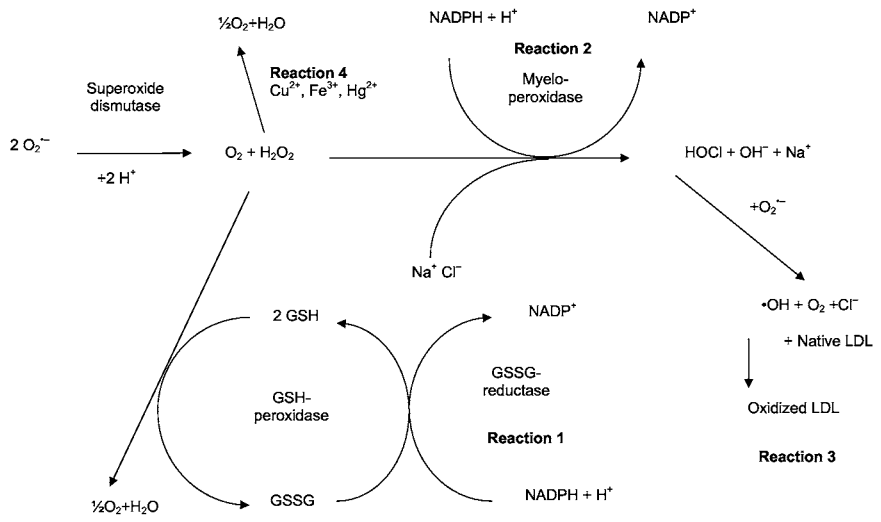

Figure 1. Generation and quenching of radicals during LDL peroxidation (**IV**). $O_2^{\bullet-}$, superoxide anion; H_2O_2, hydrogen peroxide; GSH, glutathione; GSSG, glutathione disulphide; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized form; HOCl, hypochlorous acid; OH^-, hydroxyl ion; $^{\bullet}OH$, hydroxyl radical; LDL, low density lipoprotein.

5.2 Enzymatic peroxidation

Glutathione peroxidase from bovine erythrocytes and myeloperoxidase from human polymorphonuclear leukocytes were used in these experiments. Both glutathione peroxidase and myeloperoxidase have been associated with an elevated risk of cardiovascular diseases. Glutathione peroxidase 1 from human red cells (the same enzyme as GSH-Px) has been used in the estimation of increased risk of cardiovascular events in patients with coronary artery disease.[73] Also plasma and serum myeloperoxidase levels have been used to predict risks in patients with chest pain or acute coronary syndromes.[74,75]

5.2.1 Glutathione peroxidase

The primary function of peroxidases is to catalyze the oxidation of different molecules, e.g. polyunsaturated fatty acids, by hydrogen peroxide or other molecules of the ROOH type.[76] On the other hand, the glutathione peroxidase is one of the principal defense enzymes against oxygen free radicals and it catalyzes the reduction of hydroperoxides.[77]

The activities of glutathione peroxidase (Table 5) in the presence of mercury, copper and iron were compared to the activity of pure enzyme. The most interesting result in these enzymatic assays was that Hg^{2+} decreased the activity of GSH-Px to 1/12:th of the original value (decrease of 92 % compared to pure enzyme; $p < 0.001$) and that Cu^{2+} enhanced the inhibition of GSH-Px by mercury such that there was almost no measurable activity; the activity of GSH-Px declined to 1/120:th of the original activity (decrease of 99.1 %; $p < 0.001$ for the potentiation). Copper and iron alone had no effect on the activity of GSH-Px and Fe^{3+} did not enhance the inhibition of GSH-Px by Hg^{2+} either. It is also notable that methyl mercury had no effect on the GSH-Px activity.

Table 5. Enzyme activities of glutathione peroxidase without and with different activators or inhibitors (**IV**)

Glutathione peroxidase and activator or inhibitor	n	Enzyme activity[a]: NADPH consumed (nmol/l · min)	p
Pure enzyme	16	2561 ± 65	
Enzyme and copper (Cu^{2+})	10	2727 ± 28	0.112
Enzyme and iron (Fe^{3+})	5	2576 ± 9	0.244
Enzyme and mercury (Hg^{2+})	20	207 ± 48	<0.001
Enzyme and methyl mercury (CH_3Hg^+)	10	2636 ± 14	0.400
Enzyme, copper (Cu^{2+}) and mercury (Hg^{2+})	5	22 ± 16	<0.001
Enzyme, iron (Fe^{3+}) and mercury (Hg^{2+})	5	232 ± 4	<0.001

[a] Mean ± SD.

Each enzyme measurement with mercury, copper and iron was compared with the measurement of the pure enzyme. The differences in the means of the activities between two different groups were tested by the non-parametric Mann-Whitney U-test. The statistical significance was based on two-sided tests. The differences between the groups were considered significant when the p-value was less than 0.05.

One significant point in the protective mechanism (Fig. 1) is that the glutathione peroxidase contains four selenium atoms within its active site.[38] Thus, it is not surprising that both inorganic mercury and methyl mercury *in vivo* have been found to inhibit glutathione peroxidase activity.[78-80] Also the present results confirm that the Hg^{2+}-ion, but not methyl mercury, is an effective inhibitor of glutathione peroxidase. Although copper had no direct effect on the activity of GSH-Px, an unexpected finding was that copper, but not iron, seemed to be able to enhance the inhibitory effect of mercury. At present there is no molecular explanation for this observation. The failure to observe any inhibition with methyl mercury means that this compound must be transformed into an inorganic form before it can act as an inhibitor of GSH-Px.

The experiments with GSH-Px and Hg^{2+} in fact measured the rate of the inhibition reaction of GSH-Px (Table 5). Due to the presence of the large excess amounts of Hg^{2+} and NADPH compared to GSH-Px, the reaction can be treated as a pseudo first-order reaction. Thus, for the NADPH consumption it can be written

$$d[NADPH]/dt = k_0 * [GSH\text{-}Px]_0$$

where k_0 is the zero-order reaction rate constant. For [GSH-Px] in presence of Hg^{2+} it can be written

$$[GSH\text{-}Px] = [GSH\text{-}Px]_0 \exp(-k_1 t)$$

where k_1 is the reaction rate constant describing the inhibition of GSH-Px in presence of 10 μmol Hg^{2+}. Simple manipulation of the equations leads to the equation

$$d[NADPH]_0 / d[NADPH]_{Hg} = \exp(k_1 * t)$$

where $d[NADPH]_0$ is the NADPH consumption with pure enzyme (2561 nmol/min·l in Table 5) and $d[NADPH]_{Hg}$ is the consumption in presence of mercury (207 nmol/min·l in Table 5). Introduction of the values from Table 5 indicates that the half-life time of GSH-Px in 10 μmol/l $HgCl_2$ solution is approx. 8 min. Assuming that the half-life is directly proportional to $[Hg^{2+}]$, at the upper physiological level of 0.1 μmol/l this means that the enzyme half-life is 13 hours. In the same way, it can be estimated that a copper concentration of 10 μmol/l shortens the half-life by factor of 2.

5.2.2 Myeloperoxidase

Myeloperoxidase, which is released from cytoplasmic granules of neutrophils and monocytes by a degranulation process,[81] catalyzes two reactions resulting in efficient modification of LDL.[82] One is the reaction of H_2O_2 with protein-bound or free tyrosine to the tyrosyl radical[81,83,84] which initiates lipid peroxidation, that may lead to transformation of LDL into an atherogenic particle.[85] The other reaction, used in the present experiments, is the reaction of H_2O_2 with chloride ions producing hypochlorous acid (HOCl, Fig. 1, reaction 2),[81] which is a potent oxidant that promotes free radical reactions of lipids in lipoproteins associated with the formation of lipid peroxidation products, such as hydroperoxides.[86,87] It also reacts with unsaturated fatty acids and phospholipids to form fatty acylchlorohydrins.[88]

The effects of mercury, copper and iron on the reaction rates and activities of MPO without and with LDL in the MPO-H_2O_2-Cl^- system are presented in Figures 2 and 3 and Table 6. From the reaction curves it can be observed that all the reactions are in practice complete within 15 minutes. Compared to the pure enzyme in NaCl reaction, mercury and iron tended to inhibit myeloperoxidase slightly (25 % for Hg^{2+}; $p = 0.003$ and 16 % for Fe^{3+}; $p = 0.015$) but copper could activate MPO quite effectively (57 %; $p = 0.001$). When LDL was included in the reaction mixtures, mercury had no significant effect on the reaction rate ($p = 0.084$) but both copper and iron seemed to act as activators (72 % for Cu^{2+}; $p<0.001$ and 47 % for Fe^{3+}; $p = 0.001$). Also the direct effects of mercury, copper and iron on hydrogen peroxide as well as the influence of hypochlorous acid on myeloperoxidase were examined. Only copper seemed to increase the decomposition rate of H_2O_2, from 0.14 ± 0.05 µmol/l · min ($n = 3$) of pure H_2O_2 to 0.35 ± 0.02 µmol/l · min ($n = 3$), but also this effect was marginal compared to the enzyme reactions (the rate with MPO is 1.99 ± 0.29 µmol/l · min, $n = 6$). Neither hypochlorous acid (sodium hypochlorite) had any effect on the activity of myeloperoxidase in these conditions.

Although there are a few papers where copper, iron and the MPO-H_2O_2-Cl^- system have been discussed together,[46,89,90] neither their interactions nor the effect of mercury on the system have been studied previously. The experiments displayed in Fig. 2 (see also Table 6) proposed that copper is a clear activator but mercury and iron inhibited MPO to some extent. However, the shapes of the consumption curves of copper and mercury in Fig. 2 were indicative of metal binding to the protein and therefore to achieve a system closer to the physiological conditions, the experiments were repeated in presence of LDL, which is also claimed to be a MPO product

(HOCl) acceptor.[87] Under these conditions copper and iron were clear activators for MPO (the reaction rates indicated activation of approx. 70 % and 50 %, respectively, see Table 6 and Fig. 3), while mercury had no effect.

Table 6. Reaction rates of the reaction catalyzed by myeloperoxidase without and with low density lipoprotein and different activators or inhibitors (**IV**)

Myeloperoxidase and activator or inhibitor	n	Reaction rate[a]: NADPH consumed (μmol/l · min)	p
Reaction: H_2O_2 + NaCl			
Pure enzyme	3	4.58 ± 0.22	
Enzyme and mercury (Hg^{2+})	3	3.46 ± 0.20	0.003
Enzyme and copper (Cu^{2+})	3	7.21 ± 0.32	0.001
Enzyme and iron (Fe^{3+})	3	3.85 ± 0.22	0.015
Reaction: H_2O_2 + NaCl + LDL			
Pure enzyme	3	3.97 ± 0.26	
Enzyme and mercury (Hg^{2+})	3	3.48 ± 0.26	0.084
Enzyme and copper (Cu^{2+})	3	6.83 ± 0.32	<0.001
Enzyme and iron (Fe^{3+})	3	5.82 ± 0.14	0.001

[a] Mean ± SD.
The comparisons of reaction rates between the groups were analyzed by the Independent samples t-test. The differences between the groups were considered significant when p-value was less than 0.05.

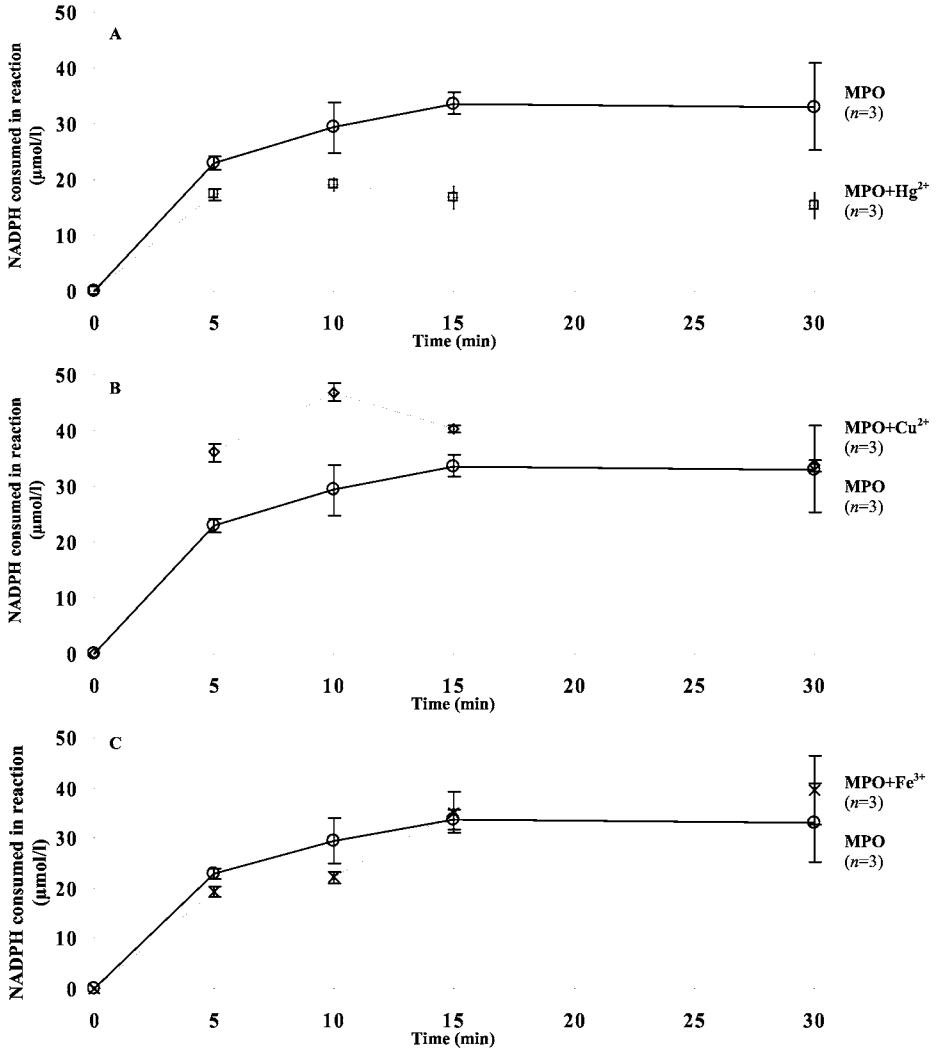

Figure 2. Consumption of NADPH (µmol/l) as a function of time in myeloperoxidase catalyzed reactions without and with mercury (A), copper (B) and iron (C) (**IV**). Sodium chloride was only used as a substrate for the enzyme in every reaction. MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.

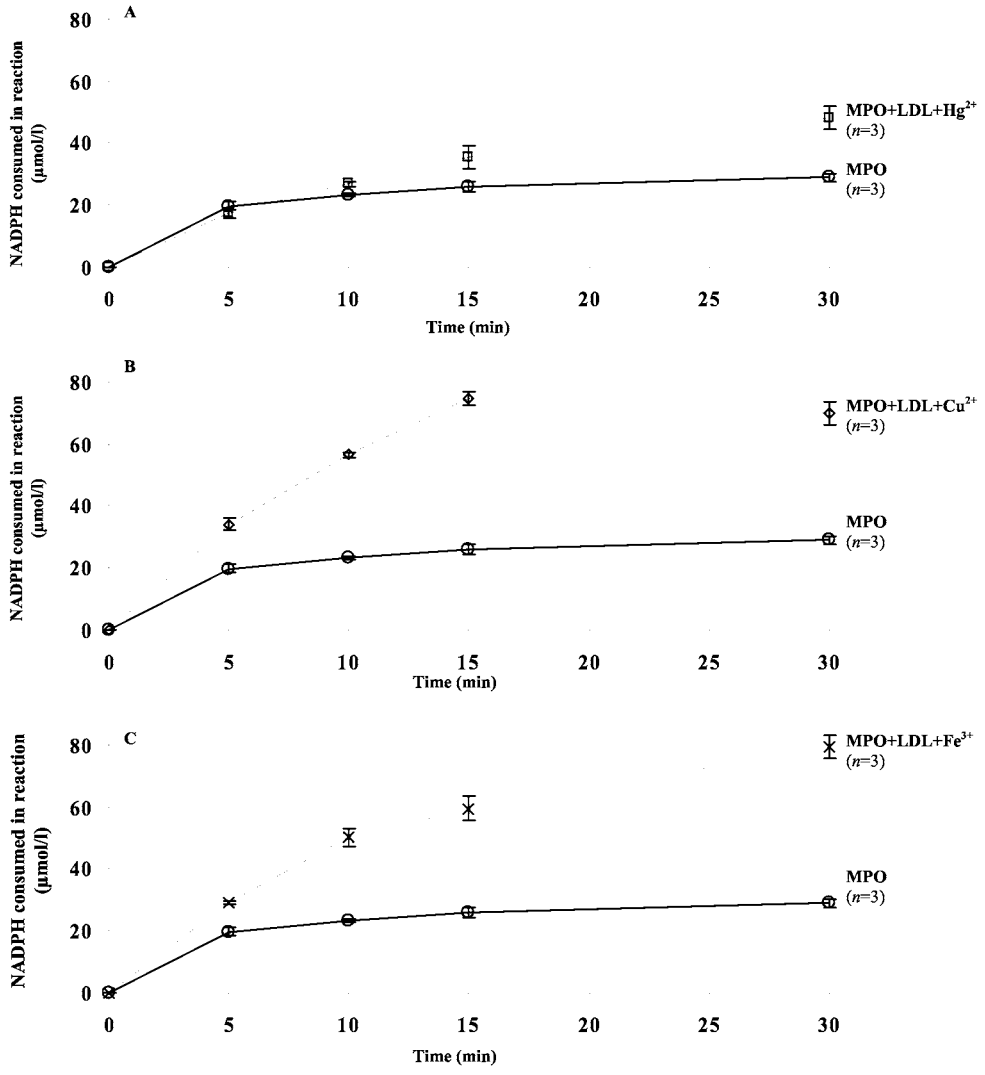

Figure 3. Consumption of NADPH (µmol/l) as a function of time in myeloperoxidase catalyzed reactions without and with mercury (A), copper (B) and iron (C) in the presence of LDL (**IV**). Each reaction mixture contained both sodium chloride and hydrogen peroxide as the substrates for the enzyme and LDL as a product acceptor. MPO, myeloperoxidase; LDL, low density lipoprotein; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.

5.3 Non-enzymatic peroxidation

In order to clarify the direct effects of mercury, copper and iron on the oxidation of LDL, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used. The LDL samples were incubated with the metal ions and MPO for 30 minutes, after which they were extracted with $CDCl_3$. The ^{1}H NMR spectra of the extracts were measured in a routine way with a Bruker AVANCE 500 DRX spectrometer operating at 500.13 MHz (Bruker-Biospin, Karlsruhe, Germany) equipped with a 5 mm inverse triple resonance probe. The spectral preparation and analyses were performed with PERCH software (PERCH Solutions Ltd., Kuopio, Finland) on standard desktop computer. Quantification was based on total-line-shape fitting method.[91] The number of lines used for fitting was adjusted so that each signal of the spectrum was well described. The final concentration of LDL was 136.8 mg/l and the effective concentration of mercury, copper or iron ions was approx. 16 μmol/l. The concentration of MPO and the other components were same as those used in the enzymatic measurements.

The NMR spectra of the LDL extracts are collected into Fig. 4. The signal areas are given using the cholesterol ester C3 protons signal (δ = 4.59 ppm) as reference. It can be seen that copper (Fig. 4) could promote non-enzymatic peroxidation of LDL since the double-bond protons signals (-C**H**=C**H**-) at δ = 5.3 ppm as well as the arachidonic and linoleic acid proton (=CH-C**H$_2$**-CH=) signals at δ = 2.79 ppm and δ = 2.75 ppm, respectively, were greatly reduced (by 35%, 65% and 82%, respectively, when compared to those of native LDL). Another measure of the oxidation is the ratio of the double-bond and linoleic acid signals. Both the signal area and ratio indicated that also iron and mercury could weakly promote the non-enzymatic peroxidation.

As to the direct reactions of the ions and MPO on LDL, the ^{1}H NMR results are evidence (Fig. 4) that copper ions as expected could effectively promote the direct non-enzymatic peroxidation of linoleic and arachidonic acid of LDL. Also iron and mercury ions seemed to have significant effects at these concentrations. In these experiments no significant interactions between MPO and the metal ions were seen. This is in disagreement with the enzymatic experiments and it will be discussed below. Nonetheless, it is obvious that mercury in realistic physiological concentrations does not seem to have any major effect on the direct non-enzymatic peroxidation of LDL. This means that the only mechanism through which mercury may promote lipid peroxidation in biological systems must occur via some indirect mechanism, for example, via the

peroxidase system. The results of the enzyme experiments are almost opposite to the ^{1}H NMR observations, in which the MPO was not influenced by the presence of the ions. Since the enzymatic experiment indicates only the production of HOCl, the ^{1}H NMR results are interpreted to signify that the reaction of HOCl and LDL is slow in comparison with the radical reactions followed in the ^{1}H NMR experiments. Nonetheless, also the enhanced HOCl production may be considered as being harmful from the point of view of lipid peroxidation.

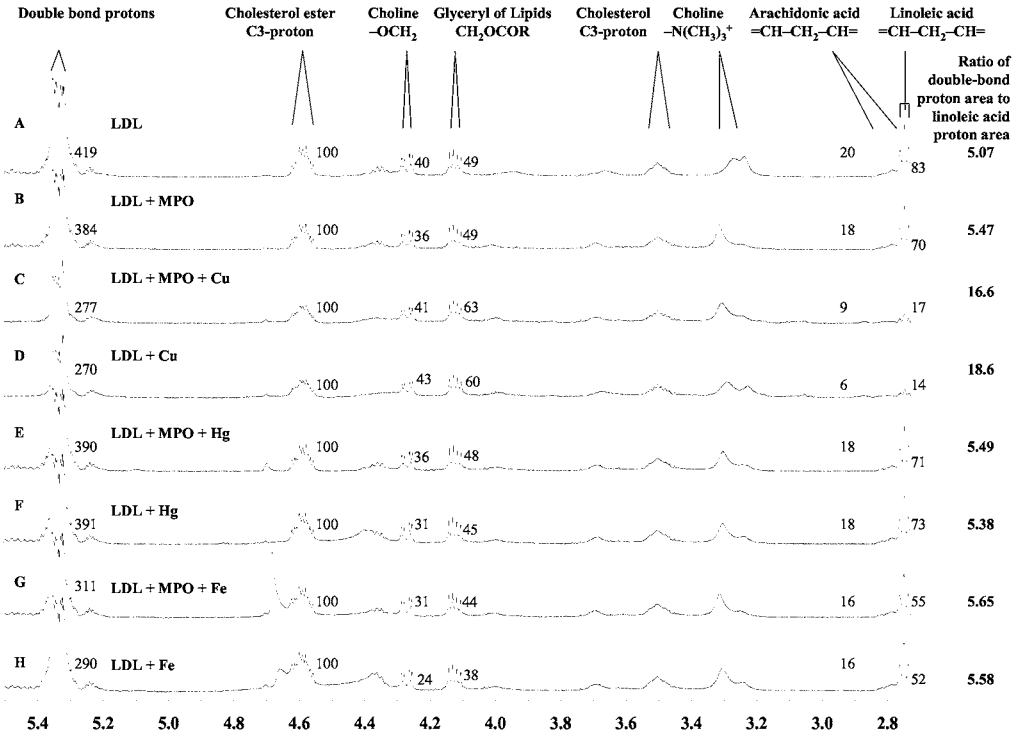

Figure 4. A part of 500 MHz ^{1}H NMR spectrum of LDL after 30 minutes incubation and extraction with $CDCl_3$ (**IV**). (A) $CDCl_3$ extracted LDL, (B) $CDCl_3$ extracted LDL + MPO, (C) $CDCl_3$ extracted LDL + MPO + Cu^{2+}, (D) $CDCl_3$ extracted LDL + Cu^{2+}, (E) $CDCl^3$ extracted LDL + MPO + Hg^{2+}, (F) $CDCl_3$ extracted LDL + Hg^{2+}, (G) $CDCl_3$ extracted LDL + MPO + Fe^{3+}, (H) $CDCl_3$ extracted LDL + Fe^{3+}. The signal assignments are given at the top of the spectra. The numbers at the signals designate their areas when the signal of esterified C3 proton of cholesterol is set to 100. MPO, myeloperoxidase. The linoleic acid/double-bond signal ratio is shown on the right.

5.4 Metal concentrations in peroxidation of low density lipoprotein

In the enzyme experiments, the final concentrations for mercury, copper or iron were 10 μmol/l, and these concentrations must be considered in relationship to those in real biological system (e.g. the blood circulation). The reference values for copper and iron in adult human blood serum are 800-1400 μg/l (13-22 μmol/l) and 800-1200 μg/l (14-21 μmol/l), respectively.[92] Thus, the concentrations of copper and iron used in this study were below these physiological ranges. Unfortunately, it is difficult to compare the real chemical activities of the elements in these solutions to those in healthy biological systems. It is probable that in healthy biological systems the activities of these two elements are somewhat lower than in these experiments. On the other hand, the mercury concentration used was much higher than the reference values, for whole blood 2-20 μg/l (0.01-0.1 μmol/l) and for blood serum <1 μg/l.[92] However, this has no impact on the conclusions. Even at a high concentration, mercury has only minor effects on the MPO reactions. Thus, the results with GSH-Px can be simply scaled down to physiological concentrations.

6. SUMMARY AND CONCLUSIONS

In this study, a molecular level model for the action of mercury in lipid peroxidation has been proposed. This model accounts for the role of mercury in LDL peroxidation and thus offers an explanation for the previous epidemiological and *in vivo* observations.[23,24,35-37] As the NMR results indicate, mercury cannot promote lipid peroxidation via a direct radical mechanism, and in this way it differs from copper, instead it is proposed that it could act via the inhibition of the enzyme, glutathione peroxidase.

The results indicate also that the effects of mercury, copper and iron on the glutathione peroxidase-myeloperoxidase system (Fig. 1) are significant in the peroxidation of LDL. The mercuric ion is a strong inhibitor of glutathione peroxidase but in presence of LDL it has no effect on myeloperoxidase. Cupric ion is a clear activator for MPO and simultaneously, it strongly enhances the inhibitory effect of mercury on the glutathione peroxidase enzyme and thus, it totally blocks the effect of GSH-Px to protect against peroxidation of LDL. Ferric ion has no effect on the activity of glutathione peroxidase but it can enhance the activity of MPO. For a proper balance of the system, the proportions of the components are critical. A simultaneous

excess of copper, iron and mercury will activate the direct peroxidation mechanism and the radical formation via MPO and will inactivate the GSH-Px defence system such that the net effect for LDL to be handled by the peroxidation pathway (reactions 2 and 3 in Fig. 1) is strongly favoured. One condition fulfilling these criteria may occur in a damaged arterial wall where a burst of copper or iron ions could be produced by an inflammatory reaction. It can be assumed that also *in vivo* mercury mainly acts via the inhibition of glutathione peroxidase enzyme. Thus, high levels of copper and iron when combined with a high exposure of mercury and a low amount of selenium in the diet form a true excess risk of coronary heart disease.

Selenium clearly has a critical role in the protection of glutathione peroxidase against mercury. The same mechanism which inactivates the glutathione peroxidase, the high affinity of mercury for selenium, functions also in the opposite direction, as documented in numerous studies,[39,64,93] i.e. selenium can sacrifice itself in the inactivation of mercury. As shown here, even a low inorganic selenium supply in the food seems to reduce the absorption of mercury into the blood but organic selenium is ineffective in this respect,[64] although it was also found to lower the mercury level in the human body.[94]

The results of this thesis suggest that a high intake of mercury from freshwater predatory fish and the consequent accumulation of mercury in the body are associated with an excess risk of coronary heart disease in eastern Finnish men. This increased risk can be explained by the action of mercury to promote lipid peroxidation.

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