THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

THE EFECT AND ROLE OF ATORVASTATIN THERAPY AND UNCOUPLING PROTEIN-2 IN LIPID METABOLISM

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Doctoral School:
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The Examination takes place at
Medical and Health Science Center, University of Debrecen
, 2010

Head of the Defense Committee:

Reviewers:

Members of the Defense Committee:

The Ph.D. Defense takes place at
The Lecture Hall of 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen
, 2010

I. INTRODUCTION

1.1 HDL

Atherosclerotic cardiovascular disease is the leading cause of death in industrialized countries. Over the last 30 years, a variety of independent risk factors of cardiovascular disease have been identified. Among them high levels of LDL (≥ 160 mg/dl vagy 4.1 mmol/L) and low levels of HDL (≤ 40 mg/dl vagy 1.03 mmol/L) are major contributing factors in the development of atherosclerosis. Three major underlying mechanism by which HDL exerts an atheroprotective effect have been proposed: i.) reverse cholesterol transport, ii.) direct effect on endothelial cells and iii.) antioxidant effect.

Currently the view is that the function and antiatherogenic activity of HDL is determined by its "quality", not by its quantity. To understand the meaning of HDL "quality" and reverse cholesterol transport, it is important to know that the HDL fraction is very heterogeneous. This heterogeneity has functional implications that may impact on the cardiovascular protective properties of the total HDL fraction.

1.2 Reverse cholesterol transport and HDL remodeling

Reverse cholesterol transport is the process where HDL carries the excess cholesterol from peripheral cells to the liver for extraction through the bile. This process contains HDL remodelling, when the small, discoid pre-β or nascent HDL, produced in the liver and small intestine, turns into the mature, spherical HDL, called HDL3. The uptake of excess free cholesterol from peripheral cells by the lipid poor, mainly apoA-I containing pre-β HDL is the first step of the reverse cholesterol transport.

Then lecithin:cholesterol acyltransferase (LCAT) coverts the free cholesterol residing on the surface of HDL to cholesterol ester, leading to cholesterol ester accumulation in the core and formation of mature, spherical HDL. Cholesterol ester in HDL is selectively taken up by the liver and steroidogenic tissues, like adrenals and gonads, through HDL receptor scavenger receptor-B1 (SR-B1). Alternatively, cholesteryl ester transfer protein can transfer cholesterol ester from HDL to apolipoprotein B-containing particles (VLDL, IDL, LDL) and in return, move triglyceride to HDL, which becomes HDL2. This way, cholesterol transferred to apolipoprotein B-containing particles is taken up by the liver through LDL-receptors. Cholesterol ester taken up by the liver is hydrolized to free cholesterol, which is either metabolized to bile acids or is eliminated directly through the bile. Continuous and extensive remodelling of HDL regulates their shape, size, composition and accounts for their heterogeneity.

1.3 Cholesteryl:ester transfer protein (CETP)

By being one of the key enzymes of reverse cholesterol transport and HDL remodelling CETP plays a central role in the regulation of the HDL level, size and shape. CETP is a HDL-associated glycoprotein and produced by the liver. CETP mediates the cholesterol ester and triglyceride exchange between HDL and apolipoprotein B-containing particles. It is yet to be clarified whether CETP is proatherogenic or antiatherogenic. CETP is considered proatherogenic, since CETP transfers cholesterol ester from the antiatherogenic HDL to the potentially atherogenic VLDL and LDL. If the CETP-mediated cholesterol ester in VLDL and LDL is taken up by arterial wall macrophages, CETP is proatherogenic. If the CETP-mediated

cholesterol ester in VLDL and LDL is returned to the liver via LDL receptor, then it is antiatherogenic.

It seems that CETP inhibitors, recently developed drugs to increase the HDL levels, support the proatherogenic effect.

1.4 Lecithin cholesterol acyltransferase (LCAT)

LCAT is the other key enzyme of the reverse cholesterol transport, which also affects the properties of HDL. In the circulation LCAT is bound to HDL. During reverse cholesterol transport LCAT and its cofactor apolipoprotein A-I catalyzes cholesterol esterification by transferring the fatty acid from sn-2 position of lecithine to the OH of cholesterol. The role of LCAT in the development of atherosclerosis is clearly antiatherogenic, since it creates free space for more cholesterol uptake on the HDL surface by enhancing the conversion of free cholesterol to cholesterol ester, and this is how it facilitates reverse cholesterol transport. Thus, LCAT beneficially modifies the plasma lipid profile by raising HDL and lowering LDL.

1.5 The antioxidant effect of HDL and the paraoxonase (PON-1)

Besides reverse cholesterol transport, the other proposed atheroprotective mechanism of HDL is its ability to inhibit LDL oxidation. This antioxidant effect of HDL is due to several molecules bound to HDL; these include apolipoprotein A-I, platelet-activating factor acetylhydrolase (PAFAH) and paraoxonase. Paraoxonase is an HDL-associated enzyme, which hydrolyses oxidized phospholipids and cholesterol esters on LDL and HDL, and protects against their accumulation on LDL. The relevance

of the protective effect against oxidative LDL is emphasized by the well known fact that oxidized LDL has a central role in the development of atherosclerosis.

Moreover, it also protects HDL from oxidation, which improves the HDL efficiency in reverse cholesterol transport. Paraoxonase is produced by the liver, and then secreted to the circulation, where it binds to HDL. Serum levels of paraoxonase in humans vary widely but are relatively constant in any given individual. The interindividual variability of paraoxonase activity is due to its polymorphism.

1.6 Atorvastatin

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the key enzyme of the intracellular cholesterol synthesis and catalyzes its first, rate limiting step, the conversion of HMG-CoA to mevalonate. This enzyme is inhibited by the statins, also known as HMG-CoA inhibitors, of which most commonly used member is atorvastatin. The statins act primarily on the liver by inhibiting de novo cholesterol synthesis, which leads to decreased cholesterol storage in the hepatocytes. This results in the overexpression of LDL receptors in the liver, which increases the removal of LDL and VLDL from plasma, and also the decreased VLDL production by the liver. Due to these processes apo B100-containing particle number decreases in plasma.

Similarly to other statins, atorvastatin drastically decreases the plasma cholesterol and LDL-cholesterol levels in a dose dependent manner in primary hypercholesterolemia, and significantly decreases the plasma triglyceride level in both primary hypercholesterolemia and primary hypertriglyceridemia.

1.7 Uncoupling protein-2 (UCP-2)

One of the main risk factors of coronary artery disease is the low HDL level, while diabetes mellitus and its complication, diabetic dyslipidemia is considered equivalent to an existing coronary artery disease in terms of risk (according to NCEP ATP III). Uncoupling protein-2 (UCP-2) plays an important role in β-cell function and insulin secretion.

The uncoupling proteins are transporters of the mitochondrial inner membrane. The outer mitochondrial membrane is permeable to smaller metabolites, while the permeability of the inner mitochondrial membrane is strictly regulated, which is necessary for maintaining the high electrochemical gradient. This gradient, created by the mitochondrial electron transport chain, is important to conserve energy and synthetize ATP. Oxidation of fuels, such as sugars, lipids, amino acids, yields electrons in the form of NADH and $FADH_2$. NADH and $FADH_2$ donate electrons to the electron-transport chain located in the mitochondrial inner membrane. As electrons move along the electron transport chain, protons are pumped from the mitochondrial matrix into the intermembrane space of the mitochondria, which establishes a proton gradient across the mitochondrial inner membrane. The energy that is established and conserved in the proton gradient is used by complex V, the ATP synthase, that synthetizes ATP. Other than the ATP synthase, there is an alternative way for the protons to get back to the mitochondrial matrix, and this is the proton leak. According to Mitchell's theory the proton leak, which is not connected to ATP synthase, is used for thermogenesis. This is how the uncoupling protein located in the brown adipose tissue was discovered, which is essential for thermogenesis, the adaptation to cold.

The cloned homologues of the UCP1 found in the brown adipose tissue were named UCP2 and UCP3. Since the exact function of the UCP2 and UCP3 is not entirely clear, several possible roles were proposed. One possibility was that UCP2 and UCP3 decreases the production of reactive oxygen species. The other possible function of UCP2 was the regulation of insulin secretion.

1.8 Insulin secretion

Insulin secretion is stimulated by intracellular signals derived from the metabolism of a variety of nutrients, the most important of which is glucose. Glucose enters the β-cells through the high capacity glucose transporter-type 2-n (GLUT-2). The glucose is processed in the glycolytic pathway, Krebs-cycle and oxidative phosphorylation, which results in ATP production. The increased ATP level results in oscillations in the ATP/ADP ratio, leading to the closure of the ATP-sensitive K^+-channel. The voltage-gated L-type Ca^{2+}-channels of the depolarized β-cells open, the cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ increases, which finally causes the exocytosis of the insulin-containing vesicles. This emphasize the importance of the tight coupling of the electron transport and ATP synthesis in mitochondria of β-cells, since the cytoplasmic ATP/ADP ratio is the central signal of the glucose stimulated insulin secretion.

1.9 Physiologic changes in fasting

Over the course of evolution the organisms had to adapt to the continuously changing nutrient supply. The response to fasting is under strict hormonal control, and the counter-regulation of insulin is in the

center of it. Thus the net affect of fasting is a switch from a fuel based on carbohydrates to one in which the energy is derived from lipid oxidation.

During starvation, triacylglycerols stored in adipose tissue is hydrolyzed to fatty acids, which are released into the circulation to supply other tissues with energy. One of the enzymes responsible for the hydrolysis of triglycerides is hormone-sensitive lipase (HSL). One of the main regulators of HSL is insulin, which inhibits HSL. After approximately 24 hours of starvation, free fatty acid concentration begins to increase in the blood, indicating that lipolysis is induced. Free fatty acids, taken up from the circulation by the liver, accumulate in hepatocytes, where they are oxidized into acetyl-CoA in the mitochondria (β-oxidation). The majority of acetyl-CoA is used for production of keton bodies (β-hydroxybutirate, acetone, acetoacetate), or, just like in other tissues, the acetyl-CoA enters the Krebs cycle to produce ATP.

1.10 The role of UCP2 in β-cell and fasting

As it was described in the previous section, the increase in the ATP levels in β-cells has a major role in the insulin secretion. UCP2 decreases the proton gradient by proton leak, which leads to decreased ATP synthesis and in the end the lower ATP level results in decreased insulin secretion. Consequently, UCP2 has a negative regulatory effect on glucose stimulated insulin secretion. This theory was supported by experiments using UCP2 knockout mice. The insulin concentration of the serum of *ucp2-/-* mice was higher, whereas the glucose levels were lower; moreover these mice in vivo secrete more insulin in response to the increase in blood glucose levels. In β-cells one of the possible regulators of UCP2 is fatty acid oxidation, since the production of reactive oxygen species is increased

during the β-oxidation of fatty acids, and UCP2 is activated by superoxids. It has been proposed that increased UCP2 expression could result in β-cell dysfunction and the development of type 2 diabetes mellitus.

UCP2 activity may be important to coordinate the physiological response of β-cells to fluctuating nutrient supply. The role of UCP2 could be important to restrict insulin secretion when blood glucose levels are low, which prevents hypoglycemia during fasting. Precise regulation is necessary to sustain the balance, that may be achieved by the exceptionally short half life of UCP2 (30 min.).

II. AIMS

2.1 The effect of atorvastatin therapy on lipid parameters and the enzymes of HDL remodeling

Since LDL cholesterol treatment goals can be achieved in the majority of patients with atorvastatin 20 mg/day, we wondered how the level of HDL would be altered by the same atorvastatin dose. Besides HDL level, HDL quality, which is responsible for the HDL antiatherogenic activity, has a crutial role in the protection against atherocslerosis. The quantity and function of HDL are principally determined by the HDL-associated enzymes and proteins, so besides apo A-I, we also examined how the activities of LCAT and CETP, enzymes of the HDL remodeling, were altered by atorvastatin 20 mg/day. Our final aim was to investigate the influence of atorvastatin on serum paraoxonase-1 concentration and activity by altering the activity of the HDL remodelling enzymes.

2.2 The effect of UCP2 on lipid metabolism in fasting

It has been previously proposed that UCP2 could play a role in the impaired insulin secretion of β-cells and the development of type 2 diabetes mellitus. Here we tested the hypothesis that the increased UCP2 in β-cells in fasting is a physiologic response in order to suppress the glucose stimulated insulin secretion, which leads to increased peripheral lipolysis and lipid metabolism in the liver. This would mean that modulation of insulin secretion by UCP2 is necessary for the fasting-induced switch from carbohydrate to fatty acid oxidation. We tested our hypothesis on *ucp2*$^{-/-}$ and wild type mice, and we were interested to see the effect of the lack of UCP2 on lipid metabolism after 24 and 72 hours of fasting.

III.METHODS

3.1 Patients

We examined the impact of atorvastatin therapy on 33 patients with types II.a and II.b primary hyperlipoproteinemia. 17 men and 16 women were enrolled, their mean age was 62.9 ± 5.55 years, and their body mass index was 26.9 ± 2.86 kg/m^2. The patients were enrolled into our study after a 6-week drug wash out period. During this period, they followed the NCEP Step 1 diet. On this regimen, the daily ingestion of cholesterol must be below 300 mg, the energy derived from fat ought not to exceed 30% of the daily caloric intake with less than 10% of this saturated fat. Subsequently, the patients received atorvastatin, 20 mg daily, for 3 months, resuming the standard diet.

The patients were not on any other medications and did not have any other definitive diseases. Inclusion criteria were: age between 21 and 70 years and previously untreated type II.a and II.b hyperlipidemia. The diagnostic criteria for type II.a and II.b hyperlipidemia was: LDL cholesterol >4.2 mmol/L with or without triglyceride >2.2 mmol/L. Exclusion criteria were: diabetes mellitus, hypertension, coronary artery disease, myocardial infarction, liver disease, cholelithiasis, anticoagulant or corticosteroid or previous lipid-lowering therapy, malignant disease, microalbuminuria, serum creatinine level >130 μmol/L, pregnancy or breast-feeding or regular alcohol drinking or smoking.

The study was approved by the Ethics Committee of the University of Debrecen after all the patients had given their informed consent.

3.2 Lipid measurements

Blood samples (5 mL venous blood) were drawn after at least 12 h of fasting. Before and after the 3-month therapy we measured the cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, apo A-I, apo B100, Lp(a), oxidized LDL and the activity of paraoxonase, CETP and LCAT.

The Cobas Integra 700 Analyser (Roche, Basel, Switzerland) was used for lipid measurements. The level of LDL cholesterol was calculated indirectly by the Friedewald equation (triglyceride level < 4.5 mmol/L). The apolipoproteins were determined by an immunnephelometric assay (Orion Diagnostica kit).

The oxLDL was quantified by sandwich ELISA. We measured antibodies against oxLDL by the Wak-Chem-Med (Berlin, Germany) kit. In this test, plasma oxLDL reacted with mouse monoclonal antibody. After washing, the antibody conjugated with peroxidase against apolipoprotein B recognized the oxLDL fixed to the solid fraction. The fixed conjugation was detected by the tetrametil benzidine reaction and measured spectrophotometrically. Intra- and inter-assay coefficients of variance (CV) were 5.4% and 8.3% respectively.

3.3 Measuring the LCAT activity

We determined the LCAT activity by a commercially available kit (Roar Biomedical Inc.). Plasma was incubated with a fluorescent substrate, and the fluorescence intensity of the intact substrate was measured at 470 nm by a Hitachi F-4500 Fluorescence Spectrophotometer. The LCAT activity is calculated by measuring the change in 470/390 nm emission

intensity. The intra-assay and inter-assay coefficients of variation were <5%.

3.4 Measuring the CETP activity

CETP activity was measured by a CETP Activity kit (Roar Biomedical Inc.). This kit contains donor (synthetic phospholipids and cholesteryl ester) and acceptor (VLDL) particles. We prepared the fllowing buffer: 150 mM NaCl, 10 mM Tris and 2 mM EDTA, pH 7.4. 6 µl plasma and a 20 µl donor/acceptor mixture were added to 1000 µl buffer. The cholesterol ester transfer from the donor to the acceptor molecule mediated by CETP was quantified by measuring the increase in the fluorescence intensity of the fluorescent cholesterol-linoleate using a Hitachi F-4500 Fluorescence Spectrophotometer. The excitation was conducted at 465 nm and the emission at 535 nm. The intra-assay and inter-assay coefficients of variation were <3%.

3.5 Measuring PON activity and concentration

The serum paraoxonase activity was determined spectrophotometrically by using a paraoxon substrate (*O*,*O*-diethyl-*O*-*P*-nitrophenylphosphate; Sigma). To measure the activity, we added 50 µl serum to 1 mL Tris/HCl buffer (100 mM, pH 8.0) that contained 2 mM $CaCl_2$ and 5.5 mM paraoxon. The generation of 4-nitrophenol from paraoxon by paraoxonase at 25°C was measured at 412 nm in a Hewlett-Packard 8453 UV–Visible Spectrophotometer. We calculated the enzymatic activity by using the molar extinction coefficient (17,100 M^{-1} cm^{-1}). One unit of the paraoxonase activity was defined as the production

of 1 nmol 4-nitrophenol per minute. We calculated the PON/HDL ratio by dividing the PON activity by the HDL cholesterol concentration.
Serum paraoxonase concentration was determined by an enzyme-linked immunosorbent assay (WAK-Chemie Medical GmbH, Germany). Serum concentration of PON was determined by reference to a standard curve constructed with purified paraoxonase. The linear range of the assay was 0.17 to 1.36 μg PON/mL. Intra-assay coefficient of variation was 3.2%.

Paraoxonase-specific activity was calculated from the PON activity and the PON concentration: PON activity was divided by PON concentration and it was expressed as nmol min^{-1} μg^{-1}.

3.6 Animals

Founders of *ucp2*$^{-/-}$ mice were obtained from Dr. Bradford B. Lowell (Harvard Medical School, Boston, MA). To obtain *ucp2* knockout (*ucp2*$^{-/-}$) mice we cross-bred heterozygote mice.

For our experiment we used male, ucp2 knockout (*ucp2*$^{-/-}$) mice and their wild type littermates at the age of 12 to 14 weeks. The animals were fasted for either 24 hours or 72 hours. Control groups were fed *ad libitum* with regular chow diet. All animals had unlimited access to drinking water. The animals were harvested after24 or 72 hours of fasting. At the time of sacrifice animals were weighed, their blood glucose was measured from the tail vein. Blood was obtained by cardiac puncture for additional biochemical assays. The liver and epididymal fat pads were removed, weighed, and snap-frozen or processed for histological studies. All experiments were performed by the approval of the Lifespan Animal Welfare Committee of Rhode Island Hospital.

3.7 Biochemical measurements

We used commercially available kits to determine plasma levels of non-esterified fatty acids (Wako, Richmond, VA), triacylglycerols, and β-hydroxybutyrate (StanBio, Boerne, TX). Plasma insulin levels were measured by ELISA (Linco, St. Charles, MO). Total liver tissue lipid content was determined by the chloroform: methanol extraction method. We calculated the total lipid content by the following equation: (lipid weight in an aliquot x volume of the chloroform layer)/aliquot volume.

3.8 Histological studies

Liver tissue pieces were embedded and frozen in Tissue-Tek medium (Sakura, Torrance, CA). To prepare the slides the embedded liver tissues were sectioned at 4 μm thickness. To estimate the extent of hepatic lipid accumulation, we prepared digitized images of liver slides stained with oil-red-O (MicroPublisher 3.3 RTV, Qimaging, Burnaby, British Columbia). We recorded the area of oil-red-O staining above a constant optical density threshold using Image Pro Plus 5.1 (MediaCybernetics, Silver Springs, MD) to calculate the percentage of area stained positive for oil-red-O. Constant optical conditions were maintained along the entire morphometric evaluation.

3.9 Western Blot analysis

Liver tissues were digested in the following buffer: 50 mM HEPES (pH 7.5), 200 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1 % Tween-20, 10 % glycerol, 0.1 mM Na-orthovanadate, 1 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml apoprotinin, és 1 mM β-glycerophosphate. The protein concentration of the lysates was measured

by BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were loaded and size-fractionated by 10% SDS-polyacrilamide gel electrophoresis, then transferred to a PVDF membrane (PerkinElmer Life Sciences, Waltham, MA). The immunoblots developed using polyclonal rabbit antibodies against phosphorylated HSL (Cell Signaling, Danvers, MA), PPAR-α (Sigma), PPAR-γ and SREBP1-c (Santa Cruz, Santa Cruz, CA). Secondary donkey antibody (Santa Cruz) was conjugated with horseradish peroxidase. The immunoblots were detected by ECL (PerkinElmer). Equal loading was confirmed using β-actin.

3.10 Real time quatitative PCR

Total RNA was extracted from snap-frozen liver tissue specimens with TRIzol reagent (Invitrogen, Carlsbad, CA). Then, we used first-strand cDNA synthesis kit (Roche)for reverse transcription. Polymerase chain reaction (PCR) was performed using an iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). We utilized the TATA box-binding protein (TBP) as reference gene. The full-length mouse TBP gene (957 bp) was amplified and cloned into pCR2.1 vector to create standard curves by serial dilutions. Sample cDNAs (equivalent of 5 ng total RNA) were used as template with gene-specific primers. Each sample was normalized using its TBP mRNA content and data are given as relative abundance over fed controls.

3.11 Statistical analysis

Statistical analysis was performed by the SAS for Windows 6.11 computer program. Data were presented by descriptive analysis (mean ± standard deviation) and were evaluated by paired Student's *t* test. The normality of distribution of data was tested by the Kolmogorov–Smirnov test. Non-normally distributed parameters were transformed logarithmically to correct any skewed distributions. The significance level was adjusted to $P < 0.05$.

We presented the results of the experiments on mice as mean ± SE. The data were analyzed with unpaired Student *t* test or ANOVA when multiple comparisons were made. Association between categorical groups was evaluated by the Fisher's exact probability, Mann-Whitney U test, and binomial exact calculations. Differences with calculated *P* values <0.05 were regarded as significant.

IV. RESULTS

4.1 Lipid parameters and changes in LCAT, CETP and PON activity

First, we were interested to see the effect of atorvastatin therapy on lipid parameters. Our results demonstrate a beneficial effect of atorvastatin on lipid parameters.

From the baseline, atorvastatin significantly reduced the levels of serum cholesterol (from 6.68 ± 0.61 to 4.57 ± 0.78 mmol/L; $P<0.001$) and triglyceride (from 1.75 ± 0.77 to 1.20 ± 0.31 mmol/L; $P<0.001$). We also found a significant decrease in the levels of LDL cholesterol (from 4.39 ± 0.50 to 2.65 ± 0.54 mmol/L; $P<0.001$) and the main apolipoprotein of LDL, the apo B100 (from 1.40 ± 0.24 to 0.88 ± 0.16 g/L; $P<0.001$). Ninety-two percent of the patients achieved the NCEP ATP III LDL treatment goals. On the other hand, the atorvastatin therapy did not significantly influence the levels of HDL cholesterol (from 1.49 ± 0.29 to 1.43 ± 0.31 mmol/L) and apo A-I (from 1.649 ± 0.24 to 1.647 ± 0.21 g/L).

We examined the impact of atorvastatin on the level of oxLDL and found it to be significantly reduced (from 60.49 ± 15.94 to 32.65 ± 9.43 U/L; $P<0.001$).

Since the atheroprotective role of HDL depends not only on the HDL concentration, but also on the HDL function, we wondered how the HDL function would change after atorvastatin therapy. One of the antiatherogenic properties of HDL is its antioxidant effect, which is partly due to the HDL-associated paraoxonase, we were curious to see the effect of atorvastatin on paraoxonase. Atorvastatin had a beneficial effect on the activity of paraoxonase-1, which significantly increased (from 120.4 ±

84.1 to 145.9 ± 102.2 U/L; $P < 0.001$). However, the paraoxonase-1 concentration was not altered significantly (from 45.4 ± 2.8 to 46.8 ± 3.1 μg/mL). The paraoxonase-1-specific activity, calculated from paraoxonase-1 activity and concentration, was significantly higher after atorvastatin therapy (from 2.65 ± 0.4 to 3.11 ± 0.35 nmol min^{-1} μg^{-1}; $P<0.01$). We also investigated whether the PON activity/HDL-C ratio was altered and found it to be significantly elevated (from 84 ± 57.9 to 109.83 ± 80.43; $P<0.01$.

The aim of our study was to also examine the alterations in two key enzymes associated with HDL remodeling. Atorvastatin induced a significant increase in the activity of LCAT (from 36.78 ± 18.31 to 44.76 ± 17.43 nmol/mL/h; $P<0.05$) and a significant decrease in the activity of CETP (from 151.29 ± 11.35 to 143.59 ± 9.40 pmol/mL/h; $P<0.001$).

According to the current view, both the increase of the LCAT activity and the decrease of the CETP activity are considered antiatherogenic, which contribute to the beneficial, atheroprotective effect of atorvastatin. This suggests that atorvastatin is responsible for the protection against atherosclerosis not only by the LDL-reducing effect of the statin family, but also by some other ways.

4.2 Changes of biochemical markers after fasting

We fasted *ucp2*$^{-/-}$ mice and wild type littermates up to 72 hours to analyze the impact of UCP2 deficiency on the metabolic response to fasting.

Serum insulin levels in *ucp2*$^{-/-}$ mice remained higher even after 24-hour fast, while this difference disappeared when fasting continued for a total of 72 hours.

Although ambient blood glucose levels and fasting-induced hypoglycemia in this cohort of *ucp2*$^{-/-}$ mice were not significantly different from wild type, blood glucose levels following 72-hour fast in *ucp2*$^{-/-}$ mice were lower compared to wild type controls.

Serum levels of fatty acids increased in wild type mice after 24-hour fast, while this response was diminished in *ucp2*$^{-/-}$ mice. This genotypic difference was even more apparent after 72-hour fast, when fatty acid levels dropped below pre-fasting values in *ucp2*$^{-/-}$ mice. Following 24-hour fast, amounts of phosphorylated HSL dramatically increased in wild type mice indicating release from insulin-mediated inhibition, but this change was attenuated in *ucp2*$^{-/-}$ mice. Thus, fasting-induced lipolysis is diminished in *ucp2*$^{-/-}$ mice. 72-hour fast resulted in massive lipolysis regardless of the genotype, resulting in the disappearance of epididymal fat pads in most animals and preventing the systematic analysis of HSL activation at this extreme phase of fasting. Serum levels of triacylglycerols in wild type mice became gradually lower as fasting continued for up to 72 hours. Interestingly, this trend was delayed in *ucp2*$^{-/-}$ mice, complementing changes in serum levels of fatty acids. After 72-hour fasting serum triacylglycerols levels were uniformly low, consistent with the overall depletion of peripheral fat stores. We then assessed fasting-induced ketogenesis by measuring serum levels of β-hydroxybutyrate (BHA). While wild type mice responded with a sharp and continuous increase in BHA production, *ucp2*$^{-/-}$ mice were unable to maintain the same trend.

4.3 Hepatic steatosis

Reduced amounts of fat accumulated in livers of *ucp2*$^{-/-}$ mice after 24-hour fast as assessed by total tissue lipid extraction and oil-red-O staining. Surprisingly, however, steatosis became more severe in *ucp2*$^{-/-}$ mice after 72-hour fast when it already showed substantial resolution in wild type mice. Increased hepatic fat accumulation in *ucp2*$^{-/-}$ mice was also indicated by elevated liver weight/body weight ratios after 72-hour fast.

4.4 Changes in the expression of molecules involved in lipid regulation in the liver

Gene expression for carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzyme for mitochondrial fatty acid uptake, gradually increased with fasting in wild type mice, but showed no change in *ucp2*$^{-/-}$ mice. The pattern of gene expression was similar for medium chain-specific acyl-CoA dehydrogenase (MCAD), a key enzyme for mitochondrial β-oxidation.

Fatty acid oxidation may occur through accessory pathways during starvation. Therefore, we also analyzed expression of acyl-CoA oxidase (AOX) (peroxisomal β-oxidation) and cytochrome p450 2E1 (CYP2E1) (microsomal ω-oxidation). Both genes had lower expression in fasted *ucp2*$^{-/-}$ mice. Gene expression for mitochondrial β-hydroxy-β-methylglutaryl-CoA synthase (mtHMGS), a pivotal enzyme of ketogenesis, rose markedly upon fasting in wild type mice, but remained unchanged in *ucp2*$^{-/-}$ mice.

In addition, gene expression for microsomal triglyceride transfer protein (MTTP), the protein responsible for exporting VLDL from hepatocytes, became progressively diminished in *ucp2*$^{-/-}$ mice.

We measured mRNA levels of acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl CoA desaturase (SCD). Gene expression of all 3 lipogenic enzymes markedly diminished in both genotypes in response to fasting, although the decline in mRNA levels was more robust in *ucp2*$^{-/-}$ mice. Impairment of lipid breakdown appears to dominate the effect of UCP2 deficiency in our animal model.

Hepatic PPAR-α and PPAR-γ levels increased following a 24-hour fast in wild type mice, while this response was attenuated in *ucp2*$^{-/-}$ mice. As predicted, liver tissue SREBP-1c levels in wild type mice decreased in response to 24-hour fast, but remained unchanged in *ucp2*$^{-/-}$ mice. Notably, 72-hour fast resulted in uniformly low levels of PPAR-α, PPAR-γ, and SREBP-1c regardless of the genotype, suggesting general dysfunction of metabolic regulation at this extreme phase of fasting.

V. CONCLUSION

Of all the previous primary prevention trials, the Helsinki Heart Study was one of the firsts to reveal that the elevation of the HDL-C level by 15% might play a major role in the decrease of cardiovascular events. Subsequently, the meta-analysis of more trials showed that the elevation of HDL-C by 1% decreased the risk of cardiovascular events in women by 3% and in men by 2%. This suggests that one should consider not only reduction of the total cholesterol and LDL cholesterol caused by lipid-lowering therapy, but also a possible effect on HDL-C.

In the GREACE Study atorvastatin 20 mg/day is sufficient to achieve the treatment goals in the majority of the patients in primary and secondary prevention. In our present study, 92% of the patients achieved the LDL cholesterol treatment goals.

Besides LDL-C, the effect of atorvastatin on HDL-C is also important in assessing the progression of atherosclerosis. In our study, the levels of HDL-C and apo A-I were not altered significantly. The preventive effect of HDL on the development of atherosclerosis depends not only on its quantity, but also on its composition, which is determined by the activity of enzymes of HDL remodeling.

Previous reports stated that the elevation of LCAT increased the HDL-C level, while the level of CETP was inversely correlated with the HDL-C level. In our present study, atorvastatin 20 mg/day produced a less, but significant reduction (5%) in CETP activity.

In addition, we detected a significant elevation (21.7%) in the activity of LCAT, which also has an important effect on the HDL level and remodeling. This is one of the first reports concerning the effect of

atorvastatin on LCAT activity. However, we did not observe this, it has been previously demonstrated that atorvastatin induces an elevation in plasma apo A-I levels, probably reflecting an increase in newly formed apo A-I-containing HDL particles. Since these particles mediate reverse cholesterol transport, the greater free cholesterol content of HDL particles increases LCAT activity as LCAT continuously transforms free cholesterol into cholesterol ester.

It is interesting to note that both an increase in LCAT activity and a decrease in CETP activity have an HDL elevating effect. Despite this fact, the HDL level was not elevated significantly in our study. Besides the quantitative change of HDL, the qualitative change, which is influenced by the previously mentioned enzyme and protein, can be important. This qualitative change may influence the antiatherogenic functions of HDL such as the direct effect on the endothel, the reverse cholesterol transport and the antioxidant effect. The antioxidant effect is mainly exerted by paraoxonase that prevents the oxidation of LDL by hydrolyzing the oxidized phospholipids. Alteration of the activity of this enzyme can be one of the early indicators of the effect of LCAT and CETP on HDL-C. In our previous study, we found that atorvastatin increased paraoxonase activity, and this present study confirms our previous observation. Paraoxonase activity was significantly increased by a 20 mg daily atorvastatin therapy. This may in part contribute to a significant reduction in the proportion of oxidized LDL after atorvastatin therapy. The decreased oxidized LDL level can be also explained by the fact that the statins act primarily by inhibiting the intracellular cholesterol synthesis in the liver. The decreasing intracellular cholesterol content leads to the increased expression of hepatic LDL receptors. Thus, the effect of

atorvastatin is a marked reduction in the number of circulating LDL particles, and since there are fewer in the plasma, there is less possibility for biological modification.

In summary, the results of our study reveal that atorvastatin not only decreases the level of LDL-C, but also increases the antioxidant activity of PON. Both effects result in a marked reduction in the number of circulating oxidized LDL-C particles, which play a major role in the development of atherosclerosis.

The disorder of lipid metabolism and early atherosclerosis can develop secondarily, as part of other diseases. The foremost example is diabetes mellitus and its complication, the diabetic dyslipidemia. The fact that NCEP considers diabetes mellitus equivalent to existing coronary artery disease in terms of risk, emphasizes the significance of the early atherosclerosis caused by diabetic dyslipidemia. Today diabetes affects more and more people.

It has been argued that the rapidly growing prevalence of obesity and type 2 diabetes reflects the maladaptation of a ‘thrifty’ phenotype that originally evolved in our hunter-gatherer ancestors as a trait to promote efficient energy storage, but now it fails to meet rapid environmental changes such as continuous food availability and sedentary lifestyle. Better understanding of how the metabolic response to fasting is regulated under physiologic conditions may therefore identify molecular targets that erroneously promote this alarming trend.

In this study we identified UCP2 as a modulator of the lipid metabolic response to fasting in mice since the complex biochemical response of the liver, which involves the efficient breakdown, conversion, and redistribution of fatty acids, is perturbed in UCP2 deficiency.

Peroxisomal β-oxidation and microsomal ω-oxidation are minor pathways of fatty acid oxidation, suggested to gain significance as a result of impaired mitochondrial β-oxidation or during increased delivery of fatty acids to the liver as it occurs in fasting. While insufficient β-oxidation capacity of mitochondria in UCP2 deficiency could theoretically repartition the catabolism of fatty acids into peroxisomes and microsomes, we found no signs for such compensation.

The effect of fasting on the expression of lipid regulatory transcription factors has been well characterized. As a result of peripheral lipolysis and rapid delivery of fatty acids to the liver, fasting stimulates hepatic expression of both PPAR-α and PPAR-γ. In our experiments, 24-hour fast resulted in increased expression of hepatic PPAR-α and PPAR-γ in wild type mice, while this response was impaired in the livers of *ucp2*$^{-/-}$ mice. Notably, fasting-induced changes in hepatic SREBP-1c expression did not correlate with the degree of steatosis in our animal model. Thus, 24-hour fast resulted in decreased amounts of hepatic SREBP-1c in wild type mice, but remained unchanged in *ucp2*$^{-/-}$ mice, while genotype-specific changes in steatosis during starvation showed an opposing trend. Together with the similarly discordant expression pattern of SREBP-1c target genes ACC-α, FAS, and SCD-1, we conclude that SREBP-1c contributes little to the metabolic consequences of UCP2 deficiency in the fasting liver.

Although initially delayed, fasting-induced hepatic lipid accumulation in *ucp2*$^{-/-}$ mice becomes more severe by 72 hours, by which time steatosis is already resolving in wild type mice. It is likely that insufficient lipolysis in fasted *ucp2*$^{-/-}$ mice initially provides reduced amounts of fatty acids for hepatic uptake, accounting for less steatosis and

for diminished impact of fatty acids on metabolic regulation. When fasting continues, steatosis rapidly resolves as hepatocytes process fatty acids in order to distribute lipid-derived energy in compensation for insufficiently available glucose. In contrast, hepatic lipid utilization in response to fasting is apparently impaired in *ucp2*$^{-/-}$ mice, likely accounting for protracted steatosis.

Insulin plays a critical negative regulatory role both in fasting-induced peripheral lipolysis and in hepatic lipid breakdown. It is known from previous work that glucose stimulated insulin secretion is enhanced and hyperinsulinemia develops in *ucp2*$^{-/-}$ mice as a result of altered glucose sensing in pancreatic β cells. Here we show that residual serum insulin levels remain higher in *ucp2*$^{-/-}$ mice even after a 24-hour fast, indicating that UCP2 deficiency in β cells may impair the fasting response of hepatic lipid metabolism. These data support recent speculations on the evolutionary role of UCP2 in suppressing glucose stimulated insulin secretion of β cells and suggest that insufficient suppression of insulin secretion may perturb the fasting response of lipid metabolism in *ucp2*$^{-/-}$ mice. However, plasma insulin levels in *ucp2*$^{-/-}$ mice after 24-hour fast are comparable to non-fasting baseline levels in wild type mice and some of the metabolic changes are most prominent after 72-hour fast when fasting insulin levels in *ucp2*$^{-/-}$ and wild type mice are no longer different. Enhanced insulin signaling was recently described in the adipose tissue of mice treated with antisense oligonucleotides to UCP2. Altered peripheral insulin action may therefore contribute to changes seen in the liver of fasted *ucp2*$^{-/-}$ mice.

In conclusion, fasting-induced changes in the lipid metabolism of mice gain from the presence of UCP2. Our findings lend experimental

support to the concept that upregulation of UCP2 in β cells is a physiologically important response to fasting. Increasing UCP2 levels diminish insulin release from β cells, thus facilitating peripheral lipolysis and hepatic lipid utilization in fasting. This regulatory mechanism, however, is misdirected in type 2 diabetes where steady abundance of UCP2 may result in β cell dysfunction, thus contributing to profound metabolic disturbances. There is growing evidence that inhibition of UCP2 by genetic ablation, antisense oligonucleotides, RNA interference, or the herbal derivative genipin may restore the insulin-secreting ability of β cells and improve type 2 diabetes. Whether one needs to be concerned about the loss of physiologic effects of UCP2 under these conditions, however, remains to be seen.

VI. ABSTRACT

The aim of our study was to examine the influence of atorvastatin on lipid parameters, particularly on HDL, and on the activity of LCAT and CETP and how they affect the activity of the HDL-associated antioxidant enzyme paraoxonase. Thirty-three patients with types II.a and II.b primary hyperlipoproteinemia were enrolled into our study. The patients received atorvastatin, 20 mg daily, for 3 months. We measured the serum paraoxonase activity and concentration, oxidized LDL, LCAT and CETP activities. Atorvastatin significantly reduced the levels of cholesterol, triglyceride, LDL-C and apoB, while it did not influence the levels of HDL-C and apo A-I. The increases in serum PON-specific activity, PON/HDL ratio and LCAT activity were significant, while oxLDL and CETP activities were significantly decreased. Atorvastatin may influence the composition and function of HDL, thereby possibly increasing the activity of paraoxonase and preventing atherosclerosis.

Uncoupling protein-2 (UCP2) regulates insulin secretion by controlling ATP levels in β cells. While UCP2 deficiency improves glycemic control in mice, increased expression of UCP2 interferes with glucose-stimulated insulin secretion. These observations link UCP2 to β cell dysfunction in type 2 diabetes with a perplexing evolutionary role. We found higher residual serum insulin levels and blunted lipid metabolic responses in fasted *ucp2*−/− mice, supporting the concept that UCP2 evolved to suppress insulin effects and to accommodate the fuel switch to fatty acids during starvation. In the absence of UCP2, fasting initially promotes peripheral lipolysis and hepatic fat accumulation at less than expected rates, but culminates in protracted steatosis indicating diminished

hepatic utilization and clearance of fatty acids. We conclude that UCP2-mediated control of insulin secretion is a physiologically relevant mechanism of the metabolic response to fasting.

Keywords: Atorvastatin; High-density lipoprotein (HDL); Lecithin:cholesterol acyltransferase (LCAT); Cholesteryl ester transfer protein (CETP); Paraoxonase (PON)Prolonged fasting, lipolysis, steatosis, insulin secretion

VII. PUBLICATIONS

Publications that the thesis was based on:

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