INTRODUCTION

1.1. Lipoprotein abnormalities in atherosclerosis, the role of high-density lipoprotein (HDL)

The inverse relationship between plasma levels of HDL and coronary heart disease (CHD) has been demonstrated in a number of observational epidemiological studies, such as Framingham Heart Study, and Prospective Cardiovascular Münster Study (PROCAM), as well as in several intervention studies, for example Familial Atherosclerosis Treatment Study (FATS), which showed that increased HDL concentrations independently predicted lowered risk of CHD. Results of the High-Density Lipoprotein Intervention Trial (VA-HIT study) also suggest that the rate of coronary events may be reduced by raising HDL cholesterol and lowering triglyceride concentration independent of LDL cholesterol levels in subjects with CHD whose primary lipid phenotype was low HDL-- high triglyceride levels.

HDL, protection against atherosclerosis:

a) Reverse cholesterol transport. HDL transfers cholesterol from peripheral tissues back to the liver.

b) Protective effect of HDL on LDL oxidation. Biologically active lipids in LDL are formed in a series of three steps. The first step is the seeding of low-density lipoprotein (LDL) with products of the metabolism of linoleic acid and arachidonic acid as well as with hydroperoxides. The second step is trapping LDL in the subendothelial space and the accumulation in LDL of additional reactive oxygen species derived from artery wall cells. The third step is the nonenzymatic oxidation of LDL phospholipids that induce monocyte binding, chemotaxis, and differentiation into macrophages. Normal HDL and apoA₁ inhibit all three steps in the formation of MM (minimally modified)-LDL. Paraoxonase, a HDL-associated enzyme, prevents LDL oxidation by hydrolyzing lipid peroxides, cholesterol linoleate hydroperoxides, and hydrogen peroxide. LCAT also prevents the accumulation of oxidized lipids in LDL.
c) **Pleiotropic effects of HDL on the vascular endothelium.** HDL down-regulates expression of adhesive molecules on the surface of vascular endothelium, thus being anti-inflammatory. HDL prevents inhibition of nitric-oxide synthase by oxidized LDL, apparently helping to maintain normal vessel function. In addition to HDL inhibits platelet aggregation and thus has anti-thrombotic properties. HDL activates a number of intracellular signalling events, which can potentially lead to stimulation of cholesterol-efflux from macrophages and foam cells.

1.2. **Paraoxonase and atherosclerosis:**

Paraoxonase-1 (PON1) is a glycoprotein of 354 amino acids with a molecular mass of 43 kDa. In serum, it is almost exclusively located on HDL. PON1 can bind reversibly to organophosphate substrates, which it hydrolyzes.

The PON1 gene is located on the long arm of chromosome 7 between q21.3 and q22.1 with other members of its superfamilly. Next to the PON1 gene is a gene that codes for 1 of the pyruvate dehydrogenase kinases and may explain the linkage of PON genotypes with diabetic glycemic control in some studies. The product of PON2 has not yet been identified in biological tissue, but the PON3 gene product has recently been identified as a lactonase located on rabbit HDL.

PON1 has two amino acid polymorphisms, one at position 55 (methionine/leucine, M/L) and the other at position 192 (arginine/glutamine, R/Q). Numerous studies have been conducted to determine whether people with the PON1 192 R alloenzyme are more prone to CHD than are those with the Q alloenzyme. A meta-analysis of genetical studies reveals a statistically significant overall association between PON1 192 R allele and the presence of CHD if the Q alloenzyme of PON1 is more protective against CHD than is the R alloenzyme. There are also reports that the PON1 R allele increases the likelihood of CHD occurring by increasing susceptibility to other established risk factors, such as diabetes mellitus, cigarette smoking, and age. Some other studies have also shown an association between the PON1 55 L allele and atherosclerosis, but the literature is controversial. Low serum PON activity independent of genotype has been reported with diseases, which are known to be associated with CHD, such as diabetes mellitus, hypercholesterolemia and renal failure.

There is considerable evidence that the antioxidant activity of high density lipoprotein (HDL) is largely due to the PON-1 located on it. The N-terminus of paraoxonase associates with HDL and is stabilized by apoA1 and apoJ. Because PON can enter the intravascular space with HDL, it may therefore have access to the interstitium and areas of LDL accumulation.
and oxidative damage, and could retard the development of atherosclerosis. Previous studies showed a reduction in HDL-associated PON activity in patients with diabetes mellitus, myocardial infarction, chronic renal failure and familial hypercholesterolemia.

1.3. Dyslipidemia associated with type 2 diabetes mellitus

Diabetic dyslipidemia is characterized by high triglyceride levels, low HDL-cholesterol levels, small-dense LDL particles, and high free fatty acid (FFA) levels. This combination of features is known by many designations, including atherogenic dyslipidemia, dyslipidemia of insulin resistance, or the atherogenic lipoprotein phenotype. It contributes to the 2- to 4-fold excess risk for cardiovascular disease observed in patients with type 2 diabetes mellitus compared with nondiabetic individuals.

1.4. Lipoprotein abnormalities in uremic patients

Patients with end-stage renal disease suffer from a secondary form of complex dyslipidemia consisting of both quantitative and qualitative abnormalities in serum lipoproteins resulting from alterations in lipoprotein metabolism and composition. The prominent features of uremic dyslipidemia are an increase in serum triglyceride levels and low HDL-cholesterol levels. LDL cholesterol often is normal, but the cholesterol may originate from the atherogenic small and dense LDL subclass. The apolipoprotein B (apoB)-containing part of the lipoprotein may undergo modifications (enzymatic- and advanced glycation end-product peptide modification, oxidation, or glycosylation). Reverse cholesterol transport is impaired due to low lecithin:cholesterol acyltransferase (LCAT) and PON activity. The composition of HDL may also be altered during states of inflammation.

1.5. Dyslipidemia after renal transplantation

Kidney graft survival has greatly improved during the past decade, reaching 70-80% over the last 3 years. Cardiovascular disease is one of the main causes of death in kidney-transplanted patients. Numerous factors have been reported to contribute to posttransplant hyperlipidemia, including: steroids, cyclosporine, level of renal function, diabetes mellitus, presence and degree of proteinuria. Oxidative modification of LDL is a key event in early atherogenesis, which contributes to cholesterol accumulation in the arterial wall and the development of atherosclerotic lesions. Oxidized LDL possesses additional atherogenic properties, which include cytotoxicity and stimulation of thrombotic and inflammatory events in the arterial wall.
AIMS
To determine of serum lipoprotein and lipid levels and paraoxonase activity in disorders associated with secondary dyslipidemia and accelerated atherosclerosis, such as uremia, kidney transplantation and type 2 diabetes mellitus. There is also considerable interest in the potential pharmacological effects on PON1 activity of the lipid-lowering drugs: different fibric acid derivatives and statin therapy.

METHODS

Blood Sampling
After an overnight fast (and before dialysis in the case of uremic patients) 5 ml venous blood was obtained. The lipid parameters were determined from fresh sera. Sera for paraoxonase activity measurements were kept at -20 °C before analysis.

Lipid measurements
Serum cholesterol and triglyceride were assayed using a Boehringer Mannheim GmbH Diagnostica enzyme kit, while the HDL cholesterol was investigated by the phosphotungstic-magnesium precipitation method. The LDL cholesterol fraction was calculated indirectly using the Friedewald equation (at triglyceride levels below 4.5 mmol/l). Apolipoprotein examination was performed by the immuno-nephelometric assay in which the Orion Diagnostica kit was used.

Analysis of paraoxonase activity
Paraoxonase activity was determined using paraoxon (O,O-diethyl-O-p-nitrophenylphosphate; Sigma Chemical Co.) as the substrate by measuring the increase in the absorbance at 412 nm due to formation of 4-nitrophenol. Activity was measured by adding 50 μl serum to 1 ml Tris/ HCl buffer (100 mmol/l, pH=8.0) containing 2 mmol/l CaCl₂ and 5.5 mmol/l paraoxon. The rate of generation of 4-nitrophenol was determined at 412 nm, 25°C, by the use of a Hewlett-Packard 8453 UV-Visible Spectrophotometer. Enzymatic activity was calculated from the molar extinction coefficient 17100 M⁻¹ cm⁻¹. One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions.
Arylesterase assay

Arylesterase activity was also measured spectrophotometrically. The assay contained 1 mM phenylacetate in 20 mM Tris/HCl (pH 8.0). The reaction was started by the addition of serum and the increase in absorbance was read at 270 nm. Blanks were included to correct the spontaneous hydrolysis of phenylacetate. Enzyme activity was calculated using the molar extinction coefficient 1310 M⁻¹cm⁻¹. Arylesterase activity is expressed in units per milliliter. One unit is defined as 1 μmol phenylacetate hydrolyzed per minute.

Paraoxonase phenotype distribution

The phenotypic distribution of paraoxonase activity was determined by the dual substrate method, using paraoxon and phenylacetate substrate as well.

ELISA for serum PON concentration

Serum paraoxonase concentration was determined by enzyme-linked immunosorbent assay (WAK-Chemie Medical GmbH, Germany). Serum concentretaion of PON was determined by reference to a standard curve constructed with purified PON.

Statistical methods

The statistical analysis (regression and correlation analysis) was performed by the SAS™ for Windows™ 6.11 computer program. Data were presented by descriptive analysis (case number, mean, standard deviation). The comparisons between groups were performed by unpaired t-test and ANOVA. The p<0.05 probability was accepted as the significance level.

RESULTS

1. The aim of our study was to determine whether paraoxonase activity or phenotype is altered in patients with chronic renal failure, and in hyperlipidemic subjects without renal insufficiency, and to compare them with healthy controls. We investigated the serum paraoxonase activity and polymorphism in 119 hemodialysed uremic patients, 107 patients with primary hyperlipoproteinemia, and in 110 healthy control subjects. Serum paraoxonase activity was significantly decreased both in hyperlipidemic (p<0.01) and uremic patients (p<0.001) compared to controls. On comparison the serum paraoxonase activity was significantly lower (p<0.001) in uremic patients than in hyperlipoproteinemic
subjects. The HDL and apoA$_1$ levels in the studied groups were as follows: uremic <hyperlipidemic<control. To assess whether the observed reduction in paraoxonase activity was due to HDL and apoA$_1$ level decreases, we standardised the enzyme activity for HDL and apoA$_1$ concentrations. We found that the standardised paraoxonase activity (PON/HDL) was also lower in the uremic patients compared to the hyperlipidemic patients and controls. The standardised values for apoA$_1$ showed a similar tendency: PON/apoA$_1$: uremic patients 89.64±47.8, hyperlipidemic patients 128.12±69.83, controls 161.40±47.35. The phenotypic distribution of paraoxonase did not change significantly in different patient groups. These results suggest that HDL-concentration and phenotypic distribution of paraoxonase may not be the only determining factors, but other undetermined factors could be involved in the enzyme activity changes.

2. Besides, the author investigated 117 uremic patients on long-term hemodialysis treatment, 115 renal-transplanted patients, and 110 healthy controls. The PON activity was significantly reduced in the uremic patients compared to controls (p<0.001), while in kidney-transplanted subjects the values were almost identical to those of controls. The different immunosuppressive drug combinations did not influence PON activity. But the PON activity for a particular unit of HDL and apoA$_1$ decreased significantly in both patient groups (p<0.001). This may promote the accelerated atherogenesis in uremic and kidney-transplanted patients.

3. Fifty-six outpatients (22 male, 34 female, mean age: 56.27±8.91 years, BMI: 28.87±4.65 kg/m$^2$, average duration of known diabetes: 5.4±3.2 years) fulfilling the WHO criteria for type 2 diabetes (non-insulin-dependent diabetes mellitus) were investigated following 6 week of treatment with the National Cholesterol Education Program (NCEP) Step 1 diet. For three months during the investigation patients received 600 mg of gemfibrozil twice daily. Inclusion criteria were: patients aged between 35-70 years with untreated hypertriglyceridemia (triglyceride >2.4 mmol/l, cholesterol <7.0 mmol/l) and being on the prescribed diet. Exclusion criteria were: liver disease, gallstones, alcoholism, anticoagulant use, corticosteroid and other lipid-lowering therapy, malignant disease, microalbuminuria, pregnancy or breast feeding, serum creatinine level above 130 μmol/l, and serum cholesterol level above 7.0 mmol/l. Our patients did not include those suffering from polyneuropathy, stroke or arteriosclerosis obliterans. Nine patients suffered from
non-proliferative diabetic retinopathy. Twenty-two patients were receiving insulin, 28 sulphonylurea drugs and 6 metformin, 24 were receiving ACE-inhibitor, 7 Ca²⁺ antagonist, 5 furosemide and 8 patients were receiving β-blocker therapy. We investigated 44 healthy, age-matched, normolipidemic volunteer control subjects (18 male, 26 female, mean age 54.66±7.75 years, BMI 27.77±4.13 kg/m², serum triglyceride level 1.66±0.55 mmol/l, serum cholesterol level 4.12±0.88 mmol/l), paraoxonase activity was 164±78 U/ml. The studied parameters had a normal distribution, except triglycerides and paraoxonase activity in the diabetic patient group. Therefore data of these two were log-transformed before using the paired t-test. The comparisons between groups were performed by Student’s paired t-test. After the 3-month investigation period serum triglyceride level was found to be significantly reduced (p<0.001) following the administration of gemfibrozil. The total cholesterol level was not significantly changed, while the protective HDL-cholesterol level was slightly increased (p<0.05). The apoA₁ level was also significantly increased (p<0.01). Serum paraoxonase activity was significantly increased following gemfibrozil therapy (p<0.001). Serum paraoxonase activity was significantly lower in patients with diabetes mellitus. After the three-months gemfibrozil administration the PON activity value came close to healthy controls, but did not reach that. To determine whether the altered paraoxonase activity was due to HDL-cholesterol or apoA₁ level increase, we standardized the enzyme activity for HDL-C and apoA₁ concentration (PON/HDL-C and PON/apoA₁ ratio). PON/HDL-C ratio (p=0.19) and PON/apoA₁ (p=0.93) were not significantly increased by gemfibrozil treatment. The paraoxonase activity did not correlate with the concentration of glycohemoglobin and the duration of diabetes. The serum levels of LDL-C, apo B, lipoprotein (a), hemoglobin A₁C and fibrinogen, the systolic and diastolic blood pressures, as well, as the BMI values did not change significantly.

4. Fifty seven hyperlipidemic patients were involved in the study (26 males, 31 females). The mean BMI was 26.17±6.17 kg/m². The effects of twice daily 600 mg gemfibrozil on serum cholesterol, lipoproteins, triglyceride, apolipoproteins and fibrinogen levels as well as on liver and kidney function were measured. During the three month study it was observed that following the effect of gemfibrozil: the serum triglyceride level (p<0.003) and cholesterol level (p<0.05) were significantly decreased, while the protective high-density lipoprotein (p=0.41) was not significantly increased. The low-density lipoprotein (p=0.37) was not significantly decreased while apolipoprotein B-100 (p<0.05) was
significantly decreased, and apolipoprotein A₁ (p=0.31) remained unchanged. The serum paraoxonase activity was increased (p<0.001). The standardized values for HDL (PON/HDL) were also increased (p<0.01). We concluded that gemfibrozil has a lipid lowering effect in hypertriglyceridaemic patients and might improve the antioxidant status by increasing serum paraoxonase activity.

5. Fifty-two type IIb hyperlipidemic patients with coronary heart disease were enrolled in the study. All of them had one or more of the following conditions in their past medical histories: documented acute myocardial infarct, coronary stenosis diagnosed by angiography, stable angina pectoris, positive ECG and thallium scintigraphy findings. Triglyceride levels were between 2.3 and 4.6 mmol/l. After six weeks on step 1 diet of the National Cholesterol Education Program (NCEP) patients (36 females, 16 males; mean age: 52.8±10.8 years, mean BMI: 27.13±5.37 kg/m²) received 1x200 mg micronised fenofibrate daily. During the three months therapy with 200 mg of micronised fenofibrate once a day serum triglyceride (p<0.001), cholesterol (p<0.001) and LDL-C (p<0.05) levels were significantly reduced while HDL-C level was significantly increased (p<0.001). The main apolipoprotein of HDL, apoA1 was significantly increased (p<0.05), while apoB100 – main apolipoprotein of LDL cholesterol was significantly reduced (p<0.01) by micronised fenofibrate therapy. HDL associated paraoxonase activity significantly increased (p<0.05), while the concentration of the enzyme did not change significantly (before treatment: 48.8±10.2 µg/ml, after treatment: 45±12.3 µg/ml). Arylesterase activity (p<0.01) significantly increased while salt stimulated paraoxonase activity (p<0.48) did not change significantly. Elevated paraoxonase activity could be the result of significantly increased HDL level, thus PON/HDL ratio was determined. This activity was significantly elevated after therapy (p<0.05). ApoA₁ is an important component of HDL for activation of PON thus PON/apoA₁ ratio was also determined. There was no significant elevation in this ratio (p=0.39). There was no significant change in serum PON concentration after therapy, suggesting that the effect of fenofibrate on lipid metabolism (apoA₁, lipoprotein lipase), which contributes to increase in enzyme activity via fibrate-induced structural changes in HDL. We conclude that micronised fenofibrate is able to normalize lipid profile in our patients and reduce the incidence of cardiovascular diseases via the LDL oxidation inhibitor effect.
6. The effect of simvastatin on lipids and paraoxonase activity was examined in 112 (52 male and 60 female) hyperlipoproteinaemic patients with Fredrickson type II. A and II. B hyperlipoproteinaemia (mean age: 52.15±7.99 year, mean BMI: 27.53±4.30 kg/m²). The effects of simvastatin 20 mg per day on serum cholesterol, lipoproteins, triglyceride, apolipoproteins as well as on liver function were measured. We found that simvastatin administration significantly decreased the serum cholesterol, triglyceride (p<0.01), LDL-C, apoB levels (p<0.05), while HDL-C and apo A1 levels did not change significantly. The HDL-associated paraoxonase activity did not change significantly after simvastatin therapy. The simvastatin did not modify the phenotypic distribution of paraoxonase. Beside the quantitative lipid lowering effects, HMG-CoA reductase inhibitors can also lead to qualitative modifications of lipoprotein particles. It can be assumed that the HDL-associated paraoxonase enzyme activity may be altered as a result of the qualitative modification of HDL. The short term simvastatin administration may not be effective on HDL associated antioxidant PON activity.

CONCLUSION

Additional information is required particularly about nutritional and pharmacological effects on serum PON1 activity that might lead to intervention trials to test capacity to prevent atherosclerosis. Information from prospective cohort studies may also be valuable, as would a more detailed knowledge of the basic biochemistry of PON1 action and its interrelations with other HDL enzymes.

REFERENCES:


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