GEMFIBROZIL INCREASES PARAOXONASE ACTIVITY IN TYPE 2 DIABETIC PATIENTS. A NEW HYPOTHESIS OF THE BENEFICIAL ACTION OF FIBRATES?

Z. BALOGH, I. SERES, M. HARANGI, P. KOVÁCS, GY. KAKUK, GY. PARAGH

SUMMARY - Objective: The constellation of elevated triglycerides and decreased high-density lipoprotein is recognised as a risk factor for CAD and constitutes the major dyslipidemia in type 2 diabetes. The high-density lipoprotein associated paraoxonase activity can inhibit low-density lipoprotein oxidation and has an antiatherogenic effect. To determine the effect of gemfibrozil on the dyslipidemia and serum paraoxonase activity in patients with type 2 diabetes.

Material and methods: Fifty-six type 2 diabetic patients with associated hypertriglyceridemia were involved. They were investigated for the effect of twice daily 600 mg of gemfibrozil on serum cholesterol, triglycerides, apolipoproteins, fibrinogen level, body mass index and glycated hemoglobin. Serum paraoxonase activity was measured spectrophotometrically.

Results: After three months, it was observed that under the effect of gemfibrozil, serum triglyceride level was significantly decreased (from median: 3.46 mmol/l quartiles: q1 = 1.79 q3 = 3.65; p < 0.001) while protective high-density lipoprotein (from 1.02 ± 0.22 mmol/l to 1.13 ± 0.28 mmol/l; p = 0.05) and apolipoprotein A1 (from 1.56 ± 0.33 g/l to 1.72 ± 0.29; p < 0.001) levels were significantly increased. Serum paraoxonase activity was found to be significantly increased (from median: 100.2 U/l quartiles: q1 = 60.1 q3 = 152.7 to median 118.7 U/l quartiles: q1 = 80.1 q3 = 171.0; p < 0.001) after gemfibrozil treatment. The total cholesterol, low-density lipoprotein, apolipoprotein B, lipoprotein (a), glycated hemoglobin and fibrinogen levels were not significantly changed during the three-month treatment period. The standardized paraoxonase activity for HDL and apo A1 were not significantly changed. Paraoxonase activity did not correlate with the concentration of glycohemoglobin and the duration of diabetes.

Conclusion: Twice daily administration of 600 mg of gemfibrozil is effective in type 2 diabetic patients with associated hypertriglyceridemia. It favorably lowers lipid levels, and improves antioxidant status by increasing serum paraoxonase activity.

Key-words: diabetes mellitus, paraoxonase, triglyceride, gemfibrozil.

RÉSUMÉ - Le gemfibrozil augmente l’activité paraoxonase chez les diabétiques de type 2 : une nouvelle hypothèse pour l’effet bénéfique des fibrates?

Objectif : L’association de triglycérides élevés et de cholestérol-HDL diminue est reconnue comme un facteur de risque cardiovasculaire et représente la principale dyslipidémie du diabète de type 2. L’activité paraoxonase associée aux HDL peut inhiber l’oxydation des LDL et a un effet antiatherogène.

Déterminer l’effet du gemfibrozil sur la dyslipidémie et l’activité paraoxonase sérique chez des diabétiques de type 2.

Matériel et méthodes : L’étude a impliqué 56 diabétiques de type 2 avec hypertriglycéridémie. L’effet du gemfibrozil à la dose de 600 mg 2 fois par jour a été évalué sur les taux sériques de cholestérol, triglycérides, apolipoprotéines, fibrinogène, sur le BMI et l’HbA1c. L’activité paraoxonase sérique a été mesurée par spectrophotométrie.

Résultats : Après 3 mois, sous gemfibrozil, le taux de triglycérides a diminué significativement (d’une médiane de : 3,46 mmol/l quartiles : q1 = 1,79 q3 = 3,65; p < 0,001) tandis que les HDL protecteurs ont augmenté significativement (de 1,02 ± 0,22 mmol/l à 1,13 ± 0,28 mmol/l; p = 0,05) de même que les apolipoprotéines A1 (de 1,56 ± 0,33 g/l à 1,72 ± 0,29; p < 0,001) tandis que les HDL protecteurs ont augmenté significativement (d’une médiane de : 118,7 U/l quartiles : q1 = 80,1 q3 = 171,0; p < 0,001) sous gemfibrozil. Les taux de cholestérol total, LDL, apolipoprotéine B, lipoprotéine (a), HbA1c et de fibrinogène n’ont pas significativement changé pendant les 3 mois de traitement. L’activité paraoxonase sérique n’était pas corrélée avec l’HbA1c ni avec la durée du diabète.

Conclusion : L’administration de 600 mg de gemfibrozil 2 fois par jour est efficace chez les diabétiques de type 2 avec hypertriglycéridémie. Il diminue les niveaux lipidiques dans un sens favorable et améliore le status antioxydant status en augmentant l’activité paraoxonase sérique.

Mots-clés : diabète sucré, paraoxonase, triglycéride, gemfibrozil.
The main causes of mortality and morbidity in type 2 diabetic patients are coronary artery disease (CAD) and other vascular diseases [1, 2]. Insulin resistance is often associated with dyslipidemia which consists of high triglyceride levels, low levels of HDL-cholesterol, elevated levels of LDL-cholesterol, VLDL and small dense LDL, all factors that predispose to CAD [3, 4].

Oxidative modification of LDL is a key event in foam-cell development and in the stimulation of thrombosis and inflammation in the arterial wall [5, 6].

Human paraoxonase/arylesterase is a Ca$^{2+}$-dependent glycoprotein that binds to the apoA$_1$ and apoJ components of HDL and has been shown to prevent oxidation of LDL by hydrolysing lipid peroxides in vitro [7, 8]. Paraoxonase (PON) has two isoforms, which arise from a glutamine (A isoform) to arginine (B isoform) substitution at position 192 in the active site of the enzyme. A second polymorphism (Leu/Met) at position 55 does not affect the catalytic activity of paraoxonase [9], however Blatter-Garin et al. found that homozygosity for the L allele was an independent risk factor for CAD in type 2 diabetic patients [10].

Previous studies have reported paraoxonase activity to be low in patients with type 1 diabetes [11], while other authors found that paraoxonase gene polymorphism is an independent cardiovascular risk factor in type 2 diabetic patients [12, 13]: in both French Caucasian and Japanese patients with type 2 diabetes, Gln$^{192}$-Arg polymorphism of the paraoxonase gene (B allele) is associated with CAD. However, conflicting results have also been reported [14].

The precise role of PON in type 2 diabetes is controversial [15]. We sought to determine if gemfibrozil was effective in correcting the dyslipidemia and improving the serum paraoxonase activity in type 2 diabetic patients.

### PATIENTS AND METHODS

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 weeks 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. During this period physical examination, blood pressure measurements, ECG, BMI and laboratory investigations were performed at the 2nd week, and the 1st, 2nd, and 3rd months.

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 atherosclerosis 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 Ca$^{2+}$ 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/m$^2$, serum triglyceride level 1.66 ± 0.55 mmol/l, serum cholesterol level 4.12 ± 0.88 mmol/l), paraoxonase activity was 164 ± 78 U/l. Their demographic data are depicted in Table I.

### Ethics committee

The study protocol was approved by the local University Medical School Ethics Committee. All patients signed the Informed Consent Form before being enrolled.

### Blood sampling

After the patients fasted overnight for a minimum of 12 hours, blood was drawn for the following laboratory parameters: hemoglobin, hematocrit, white blood cell count, liver enzymes, urea, creatinine, fibrinogen, C-reactive protein, glycohemoglobin, uric acid, bilirubin, total cholesterol, LDL, HDL-cholesterol, triglyceride, apo A$_1$, apo B, lipoprotein (a), serum paraoxonase activity. The determination of

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoA$_1$</td>
<td>apolipoprotein A$_1$</td>
</tr>
<tr>
<td>apoB$_{100}$</td>
<td>apolipoprotein B$_{100}$</td>
</tr>
<tr>
<td>apo A-II</td>
<td>apolipoprotein A-II</td>
</tr>
<tr>
<td>apo C-III</td>
<td>apolipoprotein C-III</td>
</tr>
<tr>
<td>PON</td>
<td>paraoxonase</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>Lp (a)</td>
<td>lipoprotein (a)</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>HDL-C</td>
<td>high-density lipoprotein-cholesterol</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
</tbody>
</table>
hemoglobin A1C was performed by Biorad HPLC (normal range: 4.0-6.1%). Blood was centrifuged at 1,500 g. Lipids were measured from fresh sera. Sera were kept at −20 °C before PON activity measurements.

Lipid and apolipoprotein measurements

Serum cholesterol and triglycerides were assayed using a Boehringer Mannheim GmbH Diagnostic enzyme kit, while HDL cholesterol was determined by the phospho-tungstic-acid-magnesium precipitation method. The LDL cholesterol fraction was calculated indirectly using the Friedewald formula (triglyceride < 4.5 mmol/l) (n = 48). Eight patients had serum triglyceride > 4.5 mmol/l. Apolipoprotein examination was performed by the immuno-nephelometric assay (Orion Diagnostic kit).

Paraoxonase activity

Paraoxonase activity was determined, using paraoxon (O, O-diethyl-O-p-nitrophenylphosphate; Sigma) 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 and 25 °C, using a Hewlett-Packard 8453 UV-Visible Spectrophotometer. Enzymatic activity was calculated from the molar extinction coefficient 17 100 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 [16]. The intraassay coefficients of variation (CVs) were less than 10%.

Statistical methods

The statistical analysis was performed by the SAS \(^\text{©}\) for Windows \(^\text{©}\) 6.12 computer program (SAS Institute, Cary NC 275313 USA). Data were presented by descriptive analysis (case number, mean, standard deviation and median and quartiles for non normal distributions), while the changes in lipid parameters were analysed by ANOVA. The studied parameters had a normal distribution, except triglycerides and paraoxonase activity. The comparisons between groups were performed by Student’s paired t test. A value of \(p < 0.05\) was accepted as significant.

Results

After the 3-month investigation period, serum triglyceride level was found to be significantly reduced (from median: 3.46 mmol/l quartiles: q1 = 2.92 q3 = 5.28 to median: 2.20 mmol/l quartiles: q1 = 1.79 q3 = 3.65; \(p < 0.001\)) following the administration of gemfibrozil (Fig. 1). The total cholesterol level was not significantly changed (from 6.39 ± 0.88 mmol/l to 6.02 ± 0.91 mmol/l; n.s.) (Fig. 2), while the protective HDL-cholesterol level was slightly increased (from 1.02 ± 0.22 mmol/l to 1.13 ± 0.28 mmol/l; \(p < 0.05\)) (Fig. 3). The apo A1 level was also significantly increased (from 1.56 ± 0.34 g/l to 1.72 ± 0.29 g/l; \(p < 0.01\)) (Fig. 4). Serum paraoxonase activity was significantly increased following gemfibrozil therapy (from median: 100.2 U/l quartiles: q1 = 60.1 q3 = 152.7 to median: 118.7 U/l quartiles: q1 = 80.1 q3 = 171.0; \(p < 0.001\)) (Fig. 5). 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 value, but did not reach that level. To determine whether the altered paraoxonase activity

<table>
<thead>
<tr>
<th>TABLE I. Demographic data of healthy controls and diabetic patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic patients (before treatment)</td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Age (years) /mean ± SD/</td>
</tr>
<tr>
<td>BMI (kg/m(^2))/mean ± SD/</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)/mean ± SD/</td>
</tr>
<tr>
<td>Triglycerid (mmol/l)/median, quartiles/</td>
</tr>
<tr>
<td>PON activity (U/l)/median, quartiles/</td>
</tr>
</tbody>
</table>
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 (from 121.12 ± 61.56 to 129.02 ± 70.38; p = 0.19) and PON/apoA₁ (from 79.19 ± 44.29 to 84.76 ± 43.12; 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 (Table II). Four patients had mild reversible adverse gastrointestinal reactions (abdominal distension, nausea) during gemfibrozil therapy. Liver enzymes and kidney function were not significantly changed during the gemfibrozil administration period.
Paraoxonase is a HDL associated enzyme, which inhibits LDL oxidation in vitro [7]. Patients at high risk for CAD, e.g., those with heterozygous familial hypercholesterolemia [11], type 2 diabetes [12, 13], or uraemia requiring hemodialysis [17, 18] have low paraoxonase enzyme activities, while the Gln-Arg192 polymorphism may modulate in vivo the protective influence of the enzyme against oxidative stress.

Type 2 diabetes predisposes to oxidative modifications of lipoproteins, furthermore chronic hyperglycaemia [19], and the accumulation of advanced glycation end products seems to predispose to lipid oxidation [20]. Hedrick and coworkers found that in vitro, direct glycation of purified paraoxonase enzyme protein by incubation in 25 mmol/l glucose caused a 40% reduction in enzymatic activity, and they also measured a 40% reduction in PON activity in patients with type 2 diabetes mellitus and documented CAD compared with non-diabetic subjects. The glycated PON did not inhibit monocyte adhesion to human aortic endothelial cells in vitro [21]. This predisposition may contribute to the excess risk for atherosclerosis in patients with type 2 diabetes. Nevin et al. found that paraoxonase genotype determines about 75% of the variation in paraoxonase activity [9]. Many environmental factors influence the paraoxonase activity such as smoking, alcohol consumption, physical activity, simvastatin therapy and post-prandial phase. The effects of these environmental factors on PON1 promoters and on HDL production is still not fully understood. Levieiv found that simvastatin (during on average 6.3 weeks of treatment) increases plasma levels of paraoxonase enzyme through transcriptional mechanism, via activation of the PON1 gene promoter, by 2.5-fold in HepG2 cells [22]. Tomas et al. described that the therapeutic response of PON1 activity to simvastatin had no correlation with the genotype of PON1 [23].

In the present study we investigated the effects of gemfibrozil on serum lipid parameters and paraoxonase activity in patients with type 2 diabetes.

Gemfibrozil improved lipid and apolipoprotein profiles, particularly VLDL-triglyceride and HDL-cholesterol levels in patients with dyslipidemia when administered at a total daily dose of 900 or 1200 mg [24]. The Helsinki Heart Study, a primary prevention trial, showed a 34% reduction in the incidence of CAD over 5 years with gemfibrozil treatment compared with placebo [25]. In the VA-HIT study, where patients with low HDL levels were enrolled, after 5.2 years of gemfibrozil treatment HDL increased by 6% and cardiovascular events decreased by 22% [26].

Fibrates activate specific transcription factors belonging to the nuclear hormone receptor superfamily, termed peroxisome proliferator-activated receptors (PPARs). Among the different PPARs the PPARα form, predominantly expressed in the liver, mediates fibrate action on lipoproteins. The effects of PPAR activation by fibrates include an increase of both plasma and tissue lipoprotein lipase activity by blocking the promoter of the apoC-III gene [27]. Fibrates increase the production of apoA-I and apoA-II in the liver, which contributes to the increase in plasma HDL concentrations and can improve reverse cholesterol transport. The effects of gemfibrozil on the fibrinolytic system are controversial [28-30]. Beneficial effects on more recently identified independent CAD risk factors such as lipoprotein (a) and fibrinogen have been produced with the micronised fenofibrate [31]. Our study found that the daily 1,200 mg of gemfibrozil administration did not significantly change serum lipoprotein (a) and fibrinogen levels.

Ikeda et al. described that decreased PON activity in patients with type 2 diabetes is involved in diabetic microvascular complications, such as retinopathy and
proteinuria [32]. In our study there were no patients with microalbuminuria, while only nine patients suffered from non-proliferative retinopathy, therefore we could not compare the difference in PON activity between diabetic patients with and without retinopathy. In the present study we found that in hyperlipidemic patients with type 2 diabetes the serum paraoxonase activity significantly increased after 12 weeks of gemfibrozil treatment, and the lipid profile improved. Previously Aviram et al. described that atorvastatin hydroxy metabolites, and gemfibrozil metabolite I (p-hydroxy metabolite) possess potent anti-oxidative potential, and as a result, protect LDL, VLDL, and HDL from oxidation [33]. According to Sozmen et coworkers, poor glycemic control in diabetest is strongly associated with an increase in free radicals and may also be the cause of alterations in antioxidant enzymes (mainly catalase). The current data reveal that B allele carriers of PON are more susceptible to oxidant stress and consequent diabetic complications [34]. Turay et al found that in patients with familial combined hyperlipoproteinemia decreasing activity of paraoxonase during ciprofibrate treatment was not statistically significant, but the increase of serum apo A1 level was statistically significant [35]. In the present study we could not detect significant correlation between paraoxonase activity, HbA1C and the duration of diabetes.

The increases in PON/HDL-C and PON/apoA1 ratio were not significant after gemfibrozil treatment, suggesting that the increase in PON activity was caused by the elevation of HDL and apoA1 levels probably via activation of hepatic PPARα receptors by gemfibrozil.

Fibrates markedly increased the HDL levels and as the PON is closely associated to HDL, this could explain the PON activity increase observed after gemfibrozil therapy. Fibrates could have a more marked effect on HDL levels than on HDL remodelling. The relatively small sample size could also explain the not significant gemfibrozil effect on PON/HDL-C ratio (p = 0.19) found in our study.

Naturally, we understand the limitations of our open study design, as only a double-blind, randomised design could exclude bias. Therefore the results of our open study have to be confirmed by a randomised double-blind study. The conclusions drawn from this study are limited by the relatively small number of patients (n = 56). Also the analysis of the effect of gemfibrozil on paraoxonase activity according to the paraoxonase genotypes could be very interesting.

These results still await confirmation in type 2 diabetic patients treated with different lipid-lowering drugs (other fibrates and statins). Such studies will likely open the way to novel approaches to vascular complications in diabetes mellitus.

REFERENCES


