ORIGINAL ARTICLE

The effect of simvastatin and fenofibrate on the expression of leukocyte adhesion molecules and lipopolysaccharide receptor CD14 in type 2 diabetes mellitus

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Abstract

BACKGROUND: Mixed hyperlipidemia is often associated with type 2 diabetes mellitus and contributes to atherosclerosis progression in diabetes patients. Leukocyte activation plays an important role in atherogenesis. Both statins and fibrates are used in the treatment of mixed dyslipidemia, but their specific effect on leukocyte function remains to be elucidated. We have therefore compared the effect of simvastatin and fenofibrate on several leukocyte activation markers in diabetes patients.

METHODS: Twenty patients with type 2 diabetes and mixed hyperlipidemia were sequentially treated with simvastatin (20 mg/day) and fenofibrate (200 mg/day) in a randomized cross-over study (12 weeks each treatment). We measured adhesion molecules LFA-1, VLA-4 and CD18; in addition, lipopolysaccharide receptor CD14 on monocytes was analyzed as a marker of innate immunity. Leukocyte expression of these molecules was quantified using flow cytometry. Laboratory examinations were done at baseline and at the end of each treatment. Baseline values were compared to those of 29 healthy controls.

RESULTS: Expression of integrin CD18 (in all leukocyte populations), lipopolysaccharide receptor and VLA-4 (on lymphocytes only) was significantly higher in patients than in controls. Both treatments resulted in significant decrease in CD18 and CD14 expression; LFA-1 and VLA-4 were not influenced.

CONCLUSIONS: Both simvastatin and fenofibrate had similar favorable effect on leukocyte activation markers. This result supports the use of both statins and fibrates for the treatment of mixed hyperlipidemia in patients with type 2 diabetes mellitus.

Abbreviations:

CD	- cluster of differentiation
FITC	- fluorescein isothiocyanate
HDL	- high-density lipoprotein
LFA-1	- lymphocyte function-associated antigen-1
LDL	- low-density lipoprotein
TNF-α	- tumor necrosis factor-α
VLA-4	- very late antigen-4

INTRODUCTION

Mixed dyslipidemia contributes substantially to premature atherosclerosis in patients with type 2 diabetes mellitus. Atherogenic action of dyslipidemia is mediated through diverse mechanisms, including cholesterol deposition, endothelial dysfunction and vessel wall inflammation.

Leukocyte activation plays an important role in all of these processes. Leukocytes serve as precursors for the formation of foam cells, impair endothelial dysfunction, maintain the inflammatory infiltration of the plaque and influence myointimal proliferation (Huo & Ley 2001). The infiltration of the subendothelial space with leukocytes is mediated by cell adhesion molecules, which are expressed on the surface of leukocytes and endothelial cells (Hansson *et al.* 2002; Rosenfeld, 2002; Krieglstein & Granger 2001). Various proatherogenic factors, including atherogenic lipoproteins, up-regulate the expression of these molecules (Huo & Ley 2001; Lehr *et al.* 1995; Serrano *et al.* 2001), and cholesterol reduction was demonstrated to decrease their expression (Serrano *et al.* 2001; Weber *et al.* 1997; Rezaie-Majd *et al.* 2003; Stulc *et al.* 2008; Serrano *et al.* 2009).

Leukocyte recruitment is further enhanced by non-specific proinflammatory stimuli, acting through receptors involved in innate immunity. A key molecule among these is monocyte antigen CD14, which is a principal part of lipopolysaccharide receptor complex (Palsson-McDermott & O'Neill 2004). CD14 polymorphism has been linked to vascular disease in some studies (Hermann *et al.* 2012; Unkelbach *et al.* 1999) and increased CD14 expression was observed in patients with hypercholesterolemia (Serrano *et al.* 2001).

Lipid lowering therapy effectively reduces cardiovascular risk in patients with diabetes mellitus. Statins and fibrates are used for treatment of mixed dyslipidemia. These drugs have different but complementary beneficial effects on plasma lipid and lipoprotein levels. The specific effect of statins and fibrates on leukocyte function remains is not yet clearly understood. We have therefore compared the effect of simvastatin and fenofibrate on several leukocyte activation markers in diabetes patients with mixed hyperlipidemia.

METHODS

This was a randomized, open-label, cross-over study comparing the effect of simvastatin and fenofibrate on several leukocyte activation markers in diabetes patients. We examined the expression of α-subunits of integrins LFA-1 (CD11a) and VLA-4 (CD49d), of the β-subunit of β-2 integrins (CD18), and of lipopolysaccharide receptor (CD14).

Patients with type 2 diabetes and dyslipidemia were included in the study. The subjects were over 18 years old, with serum total cholesterol >5.0 mmol/l and triglycerides >1.7 mmol/l after at least 8 weeks of a lipid-lowering diet, without any lipid-lowering medication. Patients with severe uncontrolled hypertension (blood pressure >160/100 torr), renal insufficiency (serum creatinine >150 μmol/l), hypothyroidism, malignancy, or other major disease were excluded. Antihypertensive and antidiabetic therapy was not changed during the study. The control group consisted of healthy subjects with total cholesterol <6.0 mmol/l and triglycerides <2.0 mmol/l).

At baseline, patients were randomized to receive either simvastatin (20 mg daily, Zocor, MSD) or micronised fenofibrate (200 mg daily, Lipanthyl 200M, Laboratoires Fournier) for 3 months. The lipid lowering therapy was then discontinued for a 2-month wash-out period and the treatment was then reversed for another 3 months. Clinical and laboratory examinations were performed immediately before and at the end of each treatment period. All subjects signed an informed consent; the study protocol was approved by the Local Ethics Committee.

Blood for laboratory tests was drawn after an overnight fast. Measurements of leukocyte surface molecules were performed on the day of blood sampling. The expression of leukocyte molecules was quantified by the flow cytometry (FACSCalibur, Becton – Dickinson, Mountain View, CA, USA) using single-step staining of whole blood with monoclonal antibodies as described earlier (Stulc *et al.* 2001). We used the following murine monoclonal antibodies for antigen detection: Anti-CD11a/FITC (clone MEM-25) and anti-CD18/FITC (clone CLB-LFA-1/1) from Caltag Laboratories, Burlingame, USA, anti-CD49d/FITC (clone HP2/1) and non-specific control antibody (FITC-labeled, clone 679.1Mc7) from Immunotech, Marseille, France, and antiCD14/FITC (clone 8G3) from Diaclone SAS, Bresancon, France. Routine laboratory measurements were done using automated analyzer methods. Biochemical markers of renal, liver and skeletal muscle function included serum transaminases, creatinine, creatine kinase and a full blood count.

The results are expressed as a mean ± SD. For leukocyte molecules, the Mann-Whitney U test was used to compare the differences between the controls and patients; Wilcoxon´s paired test was used to compare the values before and after treatment. Differences in the remaining variables were tested by the two-sample or paired t test as appropriate. All statistical tests are two-sided.

RESULTS

Twenty patients with diabetes and 29 control subjects were included in the study; all patients completed the study. The treatment was well tolerated; no serious side effects were noted throughout the study. Creatinine concentrations increased (by 10%) after fenofibrate, which is a known side effect of this drug. There were no other significant changes in safety laboratory parameters. Baseline characteristics of the study groups are shown in Table 1. All patients were treated with metfor-

min, which was combined with sulfonylurea derivatives in 4 patients and with insulin in 6 patients; the mean duration of diabetes was 9±5 years.

Serum levels of total cholesterol, LDL-cholesterol and of triglycerides were higher and of HDL-cholesterol were lower in the patients than in the controls. After simvastatin treatment, total and LDL-cholesterol markedly decreased (by 29% and 21%, respectively), while concentrations of HDL-cholesterol and triglycerides remained unchanged. Fenofibrate treatment resulted in marked decrease in triglycerides (26%), mild decrease in total and LDL-cholesterol and no change in HDL-cholesterol (Table 2).

In the patients, there was increased expression of integrin CD18, (in all the leukocyte populations), of VLA-4 on lymphocytes and of CD14. Both treatments resulted in significant decrease in CD18 and CD14 expression; LFA-1 and VLA-4 were not influenced (Table 2). There were no significant correlations between the expression of leukocyte surface molecules and lipid levels.

DISCUSSION

Leukocyte expression of cell adhesion molecules and lipopolysaccharide receptor CD14 was increased in diabetes patients with mixed hyperlipidemia, and reversed after simvastatin or fenofibrate therapy. Our findings support the notion of direct antiinflammatory effect of lipid lowering treatment.

Increased leukocyte expression of various cell adhesion molecules has been repeatedly described in patients with hypercholesterolemia, and statin treatment decreased their expression to some degree in most

Tab. 1. Baseline characteristics of the study subjects.

	Control subjects	Patients
Number	29	20
Sex (male/female)	9/20	12/8
Age [years]	47.8±6.5	57.5±7.0[a]
BMI [kgm^{-2}]	25.2±3.4	30.4±3.4[a]
Hypertension	6 (21%)	12 (60%)[a]
Smoking	5 (17%)	2 (10%)
CVD	0 (0%)	6 (30%)[a]
FPG [mmol/l]	5.12±0.64	10.85±4.36[a]
HbA1c [%]	5.0±0.4	9.0±1.8[a]

BMI – body mass index, CVD – cardiovascular disease, FPG – fasting plasma glucose. Hypertension, smoking and CVD are presented as the number (percentage) of patients with the condition.
[a] $p<0.05$ patients vs. controls

Tab. 2. Serum lipid levels and leukocyte expression of activation markers in the study subjects.

	Control subjects	Patients – simvastatin		Patients – fenofibrate	
		before treatment	after treatment	before treatment	after treatment
TC [mmol/l]	4.99±0.60	6.62±0.83[a]	5.19±0.75[x]	6.52±0.65[a]	5.97±0.92[x]
LDL-C [mmol/l]	2.87±0.56	4.08±0.77[a]	2.88±0.74[x]	4.18±0.88[a]	3.72±0.71[x]
HDL-C [mmol/l]	1.62±0.34	1.34±0.21[a]	1.31±0.29	1.31±0.26[a]	1.34±0.31
TG [mmol/l]	1.11±0.62	3.61±2.0[a]	3.25±1.96	4.14±2.56[a]	2.46±1.42[x]
LFA-1					
Lymphocytes	79.9±29.8	84.2±38.3	77.4±31.9	76.8±23.6	65±27.3
Monocytes	157±57	141±49	133±43	131±43	117±42
Neutrophils	35.7±10.5	33.6±12.3	33.5±12.9	31.7±10.7	29.2±10.5
CD18					
Lymphocytes	18.1±6.5	36±23.7[a]	24.4±16.7[x]	28.4±11.5[a]	19.8±8.7[x]
Monocytes	44.9±16.7	74.3±39.3[a]	55.4±26.1[x]	64.0±39	48.3±25.4[x]
Neutrophils	22.8±10.5	38.5±23.9[a]	30±16.7[x]	35.3±24.7[a]	26.6±14.4[x]
VLA-4					
Lymphocytes	20.9±3.9	25.7±7.8[a]	25.1±11.9	24.4±6.7[a]	22±6.4
Monocytes	32±10.8	28.9±8.3	26.7±8.5	29.2±8	26.1±7.9
CD14					
Monocytes	123±39	148±33[a]	109±30[x]	154±42[a]	101±33[x]

TC – total cholesterol, TG – triglycerides, HDL-C – HDL-cholesterol, LDL-C – LDL-cholesterol.
The expression of leukocyte markers in shown in the arbitrary fluorescence units. The expression of VLA-4 on neutrophils was essentially not detectable and it is therefore not shown. CD14 is expressed only on monocytes and was evaluated only on this cell type.
[a] $p<0.05$ patients vs. controls, [x] $p<0.05$ patients before vs. after treatment

studies (Lehr *et al.* 1995; Serrano *et al.* 2001; Weber *et al.* 1997; Rezaie-Majd *et al.* 2003; Stulc *et al.* 2008, Serrano *et al.* 2009). On the other hand, to our knowledge no similar study was performed investigating patients with mixed hyperlipidemia and/or the effect of fibrate treatment. Our results thus extend the previous observations obtained with statins in isolated hypercholesterolemia. Adhesion molecules are crucial for leukocyte recruitment into atherosclerotic lesions, and suppression of these molecules by statins and fibrates may thus contribute to beneficial vascular effects of lipid lowering therapy.

The effect of both treatments on cell adhesion molecules was rather mild. This could obviously be in part due to moderate lipid lowering achieved in our study, but other factors could contribute to this finding. While decreased expression of adhesion molecules was generally reported after statin treatment, the magnitude of treatment effect varied considerably between the studies, despite significant decrease of cholesterol levels. The contradictory nature of some results is likely at least in part due to the methodological differences: leukocytes are highly reactive cells, and extensive *in-vitro* manipulations (involved in some methods) lead up-regulation of adhesion molecules, which may substantially influence the results (Wautier 1999; Stulc *et al.* 2008).

Unlike cell adhesion molecules, the role of lipopolysaccharide receptor CD14 in atherosclerosis development is less well understood. Together with toll-like receptor TLR4, CD14 is and important part of lipopolysaccharide receptor complex, which plays a prominent role in innate immunity (McDermott & O'Neill 2004). Activation of this receptor leads to downstream release of inflammatory cytokines including TNF-α and interleukin-1. CD14 is expressed mainly by monocytes and macrophages, but its soluble form may confer lipopolysaccharide responsiveness to cells that otherwise do not express CD14 (Tapping & Tobias 2000). The role of CD14 in atherogenesis is not straightforward, but it is supposed that monocyte activation by non-specific stimuli could increase inflammatory infiltration of the plaque. In line with this, CD14 C(−260)T polymorphism has been linked to vascular disease in some studies (Hermann *et al.* 2012; Unkelbach *et al.* 1999). In addition, oxidized LDL particles were recently shown to upregulate CD14 (Pasini *et al.* 2007), suggesting the direct activation of lipopolysaccharide receptor pathway by proatherogenic factors. Accordingly, increased CD14 expression was observed in patients with hypercholesterolemia, which was reversed by statin treatment (Serrano *et al.* 2001). Our results further support the role of dyslipidemia and diabetes in activating the innate immunity, and the beneficial effects of lipid lowering therapy on this pathway.

The effect of statins and fibrates on vascular function and inflammatory markers has been extensively investigated, but only few studies compared these two classes of drugs directly using a randomized design (Malik *et al.* 2001; Empen *et al.* 2003; Hogue *et al.* 2008; Fichtenbaum *et al.* 2010). Statins and fibrates have different effects on plasma lipid and lipoprotein levels. Statins predominantly decrease LDL-cholesterol, while fibrates decrease triglycerides, increase HDL-cholesterol and increase LDL particle size. The beneficial effects of these drugs are thus complementary, and both are widely used in patients with mixed dyslipidemia, which is a lipid abnormality typical associated with diabetes mellitus. The direct comparison of the effect of statins and fibrates on endothelium and inflammation is therefore clinically relevant. In this respect, Malik *et al.* (2001) observed improved vascular reactivity after both drugs. In the study by Empen *et al.* (2003), atorvastatin and fenofibrate favorably influenced serum levels of several adhesion molecules albeit in a different manner. On the contrary, in a similar study by Hougue *et al.* (2008), atorvastatin consistently and significantly decreased several adhesion and other inflammatory molecules, while fenofibrate had nearly no effect. Finally, in a work by Fichtenbaum *et al.* (2010), neither pravastatin nor fenofibrate influenced serum inflammatory and thrombogenic molecules. In our study, simvastatin and fenofibrate influenced leukocyte expression of adhesion molecules and of lipopolysaccharide receptor CD14 in a similar way. Therefore, despite differences in the mechanism of action and different effect on lipid levels, our results indicate that statins and fibrates similarly influence leukocyte function, and support the use of both classes of drugs for the treatment of dyslipidemia in patients with diabetes mellitus.

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