Coagulation and inflammation in diabetes mellitus
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Anti-inflammatory and anti-coagulant effects of pravastatin in patients with type 2 diabetes

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Abstract

Objective: Type 2 diabetes is associated with increased plasma concentrations of markers of coagulation and inflammation. Different studies show that treatment with HMG-CoA reductase inhibitors ("statins") is associated with anti-thrombotic and anti-inflammatory effects in addition to its cholesterol-lowering effect. Our objective was to evaluate the effect of pravastatin (40 mg/day) on markers of coagulation and inflammation in patients with type 2 diabetes.

Research design and methods: This was an open, randomized, cross over study, designed with an 8 week intervention period. The study group was comprised of 50 patients with type 2 diabetes (median HbA1c 7.1%) and serum total cholesterol of 5-10 mmol/l. We evaluated plasma levels of fibrinogen, F1+2, D-dimer, soluble tissue factor (sTF), von Willebrand Factor antigen (vWFag) and C-reactive protein (CRP) in blood samples drawn after fasting on day 1 and after 8 and 16 weeks.

Results: Significant reductions of total cholesterol (-22%, p<0.001), LDL-cholesterol (-32%, p<0.001) and triglycerides (-10%, p<0.05) were achieved after 8 weeks of treatment with pravastatin. In addition, significant reductions of plasma levels of F1+2 (-4.4%, p<0.05), vWFag (-5.3%, p<0.05) and sTF (-3.4%, p<0.05) were observed after treatment with pravastatin. Furthermore, plasma levels of CRP were also significantly reduced (-13%, p<0.05). Levels of fibrinogen and D-dimer did not decrease after treatment with pravastatin.

Conclusion: This study indicates that pravastatin reduces levels of markers of coagulation and inflammation in patients with type 2 diabetes. These anti-thrombotic and anti-inflammatory effects of treatment with statins could play a role in the reduction of cardiovascular complications in patients with type 2 diabetes.
Introduction

Type 2 diabetes is a leading cause of vascular morbidity and death. It is often complicated by other cardiovascular risk factors such as hypercholesterolemia, hypertension, obesity and increased markers of coagulation (1) and inflammation (2). It has therefore been recommended that therapeutic prevention of cardiovascular disease in type 2 diabetes focus not only on optimal regulation of hyperglycemia but also on treatment of other cardiovascular risk factors (3; 4).

A subgroup analysis of several large randomized clinical trials (5; 6) shows that the relative risk for cardiovascular complications in type 2 diabetic patients can be reduced by 25% using aggressive treatment of dyslipidemia with hydroxymethyl-glutaryl-CoA reductase inhibitors, also known as statins. Treatment with statins may be beneficial not only because of these agents’ lipid-lowering action, but also because of their effect on inflammation, endothelial function, adhesion of leucocytes to endothelium and thrombus formation (7). Although statins have proven to be effective in the prevention of cardiovascular disease in type 2 diabetes, little is known about these so-called pleiotropic effects in patients with type 2 diabetes.

Our objective was to determine if treatment with pravastatin has potential anti-thrombotic and anti-inflammatory effects in patients with well-controlled type 2 diabetes. Therefore, we evaluated the effect of pravastatin on coagulation and inflammation markers in patients with type 2 diabetes and serum total cholesterol of 5-10 mmol/l.

Research design and methods

In this crossover trial 50 type 2 diabetic patients were studied to evaluate the effect of pravastatin on plasma coagulation and inflammation markers. A cross over design was chosen to allow treatment comparisons in one subject rather than between subjects and because the sample size needed for detection of treatment effects is smaller. Patients were recruited from the outpatient clinic of the Slotervaart Hospital (Amsterdam, The Netherlands). Men and women ages 18-80 years who were diagnosed with type 2 diabetes for at least one year and presented with serum cholesterol levels of 5.0-10.0 mmol/l were eligible for the study. Patients with acute medical conditions; surgery during the past 3 months;
deep venous thrombosis or pulmonary embolism during the previous 3 months; significant renal, hepatic; metabolic or thyroid diseases; alcohol abuse or known familial hypercholesterolemia were excluded. Included patients were not concurrently receiving other lipid lowering, antithrombotic, or hormonal treatment, but were allowed to use an acetylsalicylic acid. Patients maintained their regular diet during the study period.

An open-label, randomized, cross over design was used. One half of the subjects (group A) began with pravastatin (Selektine, Bristol Myers Squibb, Woerden, The Netherlands, 40 mg/ day), and the other half (group B) began with no treatment. Patients visited the outpatient clinic on day one, after the first period of 8 weeks, at which time pravastatin or no treatment was crossed over for another 8-week period, and after 16 weeks at the end of the study. At each visit blood samples were taken and patients' blood pressure was measured. The active treatment and its possible effects on the measured variables were presupposed to be washed out after 8 weeks. Laboratory outcomes at day 1 and at 8 and 16 weeks were compared, with each patient being his or her own control. All patients gave their informed consent, and the study was approved by the institutional Ethical Review Board of the Slotervaart Hospital, Amsterdam.

Blood sampling and laboratory methods
Blood samples were obtained by standard venepuncture between 9:00 and 11:00 A.M. after a 12-h fast. Total, HDL, and LDL-cholesterol, triglycerides and fibrinogen were determined using standard laboratory procedures within 1 h after sampling. HDL cholesterol was determined using a direct assay. Safety parameters included creatinine, alanine amino transferase (ALT), aspartate amino transferase (AST), and creatine phosphokinase (CK) were measured with standard techniques. Glycemic control was monitored by evaluating fasting glucose, measured with standard techniques, and HbA\textsubscript{1c}, determined by high performance liquid chromatography, as described elsewhere (8). Levels of high-sensitivity C-reactive protein (hs-CRP) were determined with a near infrared particle immunoassay rate methodology (Beckman, Brea, CA). Analytical sensitivity, defined as the lowest measurable concentration that can be distinguished from zero with 95% confidence, is 0.2 mg/ml. Measurements of prothrombin fragment (F1+2) (Dade Behring, Marburg, Germany), the von Willebrand factor antigen (vWFag)
(antibodies from Dako, Glostrup, Denmark), and soluble tissue factor (sTF) (American Diagnostica, Greenwich, CT) were performed by enzyme-linked immunosorbent assay (ELISA). D-dimers were measured with an automated quantitative latex particle immunoassay (BioMérieux, Durham, NC). Interleukin (IL)-12-p70, IL-1β, IL-6, IL-10 and IL-8 were measured by cytometric bead array analysis (Beckton Dickinson Biosciences, San Diego, CA). Tumor necrosis factor-a (TNFα) and IL-6 were measured with a high sensitivity ELISA (Quantikine HS human TNFα and IL-6 ELISA; R&D systems Europe, Abingdon, Oxon, U.K.).

Statistics
Results are presented as medians with 25th and 75th percentiles. After testing for normality, Student's paired t test or Wilcoxon's signed-rank test was used to compare values after a treatment or no-treatment period. The main outcome data were tested for carryover effect by comparing treatment effects between the two patients groups (Δ group A vs. Δ group B) (9). No carryover effect was determined. Thus, we pooled data from patients after the treatment period, irrespective whether they started out or ended with the treatment period, and compared those with pooled data from the no-treatment period. Spearman's rank correlation coefficient analysis was used to examine associations between measured parameters. A two-tailed P ≤ 0.05 was considered to indicate statistical significance.

Results
A total of 56 patients were randomized to begin the study with an 8-week period of either pravastatin therapy or no treatment. Of those 56, 50 patients completed the study; 4 patients stopped during the treatment period because of side effects attributed to the medication (skin and gastrointestinal complaints), 1 patient stopped during the treatment period because he had a myocardial infarction, and 1 patient stopped because lung carcinoma was detected. Baseline clinical characteristics of the 50 patients are presented in Table 1. The median age of the patients was 59 years. Patients were overweight, with a median BMI of 29 kg/m². Patients' diabetes was well controlled, with the median HbA₁c being 7.1%. In all, 62% of the patients were treated with insulin alone or in combination with oral antidiabetic agents, whereas the other 38% were treated with oral medication
Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Type 2 diabetes patients (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59 (54-64)</td>
</tr>
<tr>
<td>Sex ratio (M/F)</td>
<td>25/25</td>
</tr>
<tr>
<td>Body mass index: BMI (kg/m²)</td>
<td>28.9 (26.8-33.1)</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>6.0 (3.0-10.3)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.1 (6.4-7.7)</td>
</tr>
<tr>
<td>Insulin treatment, n</td>
<td>31</td>
</tr>
<tr>
<td>ACE-inhibitor, n</td>
<td>11</td>
</tr>
<tr>
<td>A2-antagonist, n</td>
<td>5</td>
</tr>
<tr>
<td>Acetyl-salicylic-acid, n</td>
<td>13</td>
</tr>
<tr>
<td>Current smokers, n</td>
<td>12</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>9.7 (8.2-12.2)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.3 (5.7-6.9)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>4.1 (3.6-4.6)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 (1.0-1.5)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7 (1.4-2.8)</td>
</tr>
</tbody>
</table>

n indicates number of patients. Values are medians with 25-75 range between brackets.

Serum lipids and safety parameters

The effects of therapy are shown in Table 2. Data are expressed as medians with 25th and 75th percentile. Statistically significant reductions of total cholesterol (-1.44 mmol/l (-1.9 to -1.0)), LDL cholesterol (-1.33 mmol/l (-1.74 to -0.95)) and triglycerides (-0.19 mmol/l (-0.55 to -0.08)) were achieved after treatment by pravastatin, indicating satisfactory compliance with the study medication. HDL

Table 2. Effect of pravastatin on lipids and coagulation and inflammation markers

<table>
<thead>
<tr>
<th></th>
<th>No treatment</th>
<th>After pravastatin</th>
<th>p-value</th>
<th>Δ (after treatment – after no-treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.3 (5.6, 7.0)</td>
<td>4.9 (4.1, 5.4)</td>
<td>&lt; 0.001*</td>
<td>-1.4 (-1.9, -1.0)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>4.0 (3.6, 4.6)</td>
<td>2.7 (2.4, 3.0)</td>
<td>&lt; 0.001*</td>
<td>-1.3 (-1.74, -0.95)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.2 (1.0, 1.5)</td>
<td>1.2 (1.0, 1.4)</td>
<td>0.699+</td>
<td>0.03 (-0.11, 0.01)</td>
</tr>
<tr>
<td>Hs-CRP, mg/dL</td>
<td>4.0 (2.9, 6.2)</td>
<td>3.3 (13, 4.7)</td>
<td>0.019+</td>
<td>-0.02 (-1.34, 0.27)</td>
</tr>
<tr>
<td>TNFα, pg/ml</td>
<td>2.6 (2.1, 3.8)</td>
<td>2.7 (2.2, 3.9)</td>
<td>0.967+</td>
<td>+0.0015 (-0.98, 1.5)</td>
</tr>
<tr>
<td>IL-8, pg/mL</td>
<td>6.2 (3.0, 11.7)</td>
<td>5.7 (3.0, 10.3)</td>
<td>0.956+</td>
<td>0 (-2.7, 3.9)</td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>3.2 (2.9, 3.9)</td>
<td>3.3 (2.8, 3.8)</td>
<td>0.231+</td>
<td>0 (-1.0, 3.0)</td>
</tr>
<tr>
<td>D-dimer, µg/ml</td>
<td>0.26 (0.19, 0.39)</td>
<td>0.27 (0.19, 0.45)</td>
<td>0.104+</td>
<td>-0.02 (-0.09, 0.05)</td>
</tr>
<tr>
<td>D-dimer, µg/ml</td>
<td>0.52 (0.47, 1.29)</td>
<td>0.51 (0.47, 1.18)</td>
<td>0.007+</td>
<td>-0.04 (-0.2, 0.04)</td>
</tr>
<tr>
<td>vWFag, %</td>
<td>131 (114, 162)</td>
<td>138 (103, 175)</td>
<td>0.027+</td>
<td>-7 (-12, 3)</td>
</tr>
<tr>
<td>sTF, pg/mL</td>
<td>119 (87, 158)</td>
<td>104 (64, 146)</td>
<td>0.044+</td>
<td>-4 (-45, 4.5)</td>
</tr>
</tbody>
</table>

Data are expressed as medians with 25-75% quartile. *Normal distributed variable. tNot-normal distributed variable
Anti-inflammatory and anti-coagulant effects of pravastatin

levels did not change during drug treatment. Pravastatin did not influence glycemic control: HbA1c and glucose levels remained unchanged during treatment. Treatment with pravastatin did not significantly change safety parameters CK, ALT and AST.

Markers of coagulation and inflammation
The effects of therapy on the principal study outcome markers are summarized in Table 2. A statistically significant reduction in plasma levels of hs-CRP (−0.52 mg/dL (-1.34 to 0.27)) was achieved by pravastatin treatment. The prothrombin activation marker F1+2 was slightly, but significantly lower (−0.04 nmol/L (-0.2 to 0.04)) after active treatment. The selected markers of endothelial dysfunction, vWFag (-7% (-12 to 3)) and sTF (-4 pg/mL (-45 to 4.5)) were also significantly reduced after pravastatin treatment. Despite the overall reduction in vWFag levels, the median concentration of vWFag was increased after treatment compared with before treatment (138% (103 to 175) vs. 131% (114 to 162)) because of the extreme non-normal distribution (Fig. 1).

Treatment did not significantly lower the levels of fibrinogen, D-dimer, IL-8 or TNFα. In 20% of patients IL-12-p70, IL-1β, IL-6 and IL-10 levels were detected with

Von Willebrand Factor Antigen

Figure 1. The reduction of vWFag was -5.3 % after pravastatin treatment (dotted line) inspite of increased median concentration of vWFag (NT: 131% (114, 162) vs. PRAV: 138% (103, 175)) which is possible due to the extreme non-normal distribution of vWFag. NT: after 8 weeks no-treatment; PRAV: after 8 weeks pravastatin treatment.
the cytometric bead array analysis. In this group of patients, no changes were measured after treatment with pravastatin (data not shown). We repeated the measurements of IL-6 using a high-sensitivity ELISA (R&D systems Europe) with a detection limit at ~0.05 pg/ml. IL-6 was still detectable only in 35% of patients. In the patients with detectable IL-6 levels, no changes were observed before and after pravastatin treatment (2.9 pg/ml (1.8 to 3.2) vs. 2.6 pg/ml (1.9 to 4.5) (P = 0.3).

To identify possible mechanisms for the decrease of CRP, F1+2, sTF and vWFag after pravastatin treatment, correlations with changes in other parameters were assessed. A statistically significant correlation was observed between change in F1+2 and degree of change of D-dimer (r = .534; P < 0.0001), a finding that fits the notion that thrombin generation (F1+2) is associated with fibrin formation and proteolytic cleavage (D-dimer). No correlation between change in F1+2 and changes in fibrinogen was observed. No correlations were found between reductions of hs-CRP, F1+2, sTF and vWFag and changes in total, LDL or HDL cholesterol or triglycerides. A weak correlation was observed between degree of change of vWFag and the change of hs-CRP (r = .312; P = 0.031). No other correlations were found between the observed reductions in hs-CRP, F1+2, sTF and vWFag.

Discussion
Statins comprise a group of agents that are increasingly prescribed to counteract atherosclerosis and related cardiovascular complications. Statins also show marked clinical efficacy in individuals with type 2 diabetes. Several lines of evidence suggest that the beneficial effects of statins are attributable not only to their lipid-lowering action, but also the “pleiotropic” actions of statins. Current knowledge of such pleiotropic effects is largely derived from in vitro experiments and studies in patients with hypercholesterolemia. To specifically determine the effects of pravastatin on inflammation, coagulation, and endothelial activation markers in type 2 diabetic patients, we performed the present study. Our data demonstrated that 2 months of treatment with pravastatin reduced the levels of CRP, F1+2, sTF, and vWFag. These biological alterations may have clinical significance, as type 2 diabetes is associated with increased inflammation and coagulation activity and impaired endothelial function.
CRP is a marker of inflammation; its plasma concentration level correlates with the severity and extent of the atherosclerotic process in the arterial wall and is consistently associated with prognosis in ischemic heart disease. Several studies have shown that treatment with statins lowers CRP (10-12) in hypercholesterolemic individuals and that patients with higher levels of CRP have greater benefit from treatment with statins than patients with lower concentrations (13). In our study population of type 2 diabetic patients, we observed a significant reduction in plasma levels of CRP after treatment with pravastatin. This observation confirms previous observations (14; 15) and suggests that statins have anti-inflammatory properties in type 2 diabetic patients also.

The anticoagulant potential of pravastatin was assessed by measuring two relevant markers, F1+2 and D-dimer. The F1+2 peptide fragment is released when prothrombin is converted into thrombin, with concentrations of F1+2 in plasma reflecting the amount of in vivo generated thrombin. Several studies have shown that statins reduce circulating levels of F1+2 (16);(17) and F1+2 in samples from bleeding time wounds in patients with hypercholesterolemia (18; 19). Aoki et al. (20) showed that increased platelet-dependent thrombin generation in hypercholesterolemic patients normalizes after pravastatin treatment, whereas Szczeklik et al. (18) found that simvastatin inhibits thrombin formation in bleeding time blood. Aspirin had no further effect on thrombin formation. Likewise, Dangas et al. (19) showed that pravastatin reduced ex vivo thrombus formation, whereas the reduction was attenuated in patients on aspirin. The reduction in thrombin or thrombus formation in these studies may have been secondary to an antiplatelet effect of statins (21). Our study extends the above findings to patients with type 2 diabetes, in whom we observed that pravastatin lowered levels of F1+2. A possible explanation for this reduction of in vivo thrombin formation in these diabetic patients with mild hypercholesterolemia is that like in the previous described studies with hypercholesterolemic patients, platelet-mediated thrombin formation is reduced by statin treatment. In contrast, aspirin, did not diminish thrombin production in these patients. The mechanism by which statins might influence platelet-mediated thrombin production remains unknown. In contrast to F1+2, we observed no significant reduction of the level D-dimer after pravastatin treatment. On the basis of our study we were not able to unravel the pathophysiological mechanism behind this observation.
Theoretically, a lowered cellular sTF production or exposure may also be responsible for reduced thrombin production. At this stage, it remains uncertain whether a reduced level of sTF, as observed in our study, may translate into lower thrombin production, because the role of sTF as a stimulus of coagulation has not been established. The small decrease in sTF should probably be interpreted as diminished proteolytic cleavage from injured endothelial cells, which would support the concept of stabilization of endothelial cell function by statins (22).

The level of circulating vWFag is another marker that is considered to reflect endothelial injury. VWF is a glycoprotein stored in endothelial cells and secreted into the circulation. It increases in parallel with the degree of endothelial cell damage. In our patients the basal levels of vWFag were quite high (median 131%), a finding that might reflect the longer term vascular perturbation inflicted by type 2 diabetes (median duration of type 2 diabetes in these patients was 6 years). The observed decrease of vWFag during treatment, confirming observations on statins in patients with hypercholesterolemia (17; 23; 24), could be explained by an endothelial improving-effect of pravastatin. Data on endothelial cell-improving effects of statins in type 2 diabetic patients are scarce and conflicting. Endothelial cell-mediated vasoreactivity improves in diabetic rats after treatment with pravastatin or cerivastatin (25; 26). In a study with diabetic patients, vasoreactivity improved as soon as after 3 days of treatment with cerivastatin. In addition, the plasma level of soluble vascular adhesion molecule-1, a plasma marker for endothelial dysfunction, was decreased in these patients after 3 months of treatment (14). However, another study reported the absence of any effect on nitric oxide-dependent vasoreactivity in type 2 diabetic patients after 4 weeks of aggressive lipid-lowering treatment with atorvastatin (27). To our knowledge, our study is the first to show that pravastatin reduces levels of vWFag and sTF in diabetes patients.

Fibrinogen has been claimed as an independent cardiovascular risk factor, and increased levels of fibrinogen have been observed in patients with various atherosclerotic diseases. One study in patients with poorly controlled diabetes showed a decrease of fibrinogen after treatment with pravastatin (28). In the present study no change of fibrinogen was found after two months treatment. This finding is in line with the majority of studies in hypercholesterolemic patients, where no reduction in fibrinogen levels after statin treatment has been observed. Some of the effects of pravastatin on thrombin formation and endothelial function
may be induced by an anti-inflammatory action of this class of agents. Statins are able to block the synthesis of important isoprenoid intermediates, which serve as lipid attachments for a variety of intracellular signaling molecules. To identify a mechanism for the observed changes in inflammation, coagulation, and endothelial function after pravastatin treatment, correlations with reduction of lipids were assessed. The fact that no significant correlation was found between reductions in CRP, F1+2, vWFag and sTF and the reduction of lipids, supports the concept that effects other than lipid-lowering action play a role in these changes. In addition, the change of vWFag after pravastatin treatment was associated with the degree of change of CRP, suggesting that the endothelial cell-improving effect was attributable to an anti-inflammatory and not a cholesterol-lowering effect of pravastatin. We speculated that the regulation of vWFag and sTF were related, both being markers of endothelial cell dysfunction. However, we did not find an association between changes of both parameters. This might be explained by a different pattern of cleavage, secretion or elimination from the circulation.

In conclusion, our data demonstrate that treatment with pravastatin for 2 months induces anti-inflammatory, anti-thrombotic and endothelial-improving actions in patients with type 2 diabetes and mild hypercholesterolemia. These findings provide an additional biological basis for the presumed importance of pleiotropic effects of statin treatment in patients with type 2 diabetes and cardiovascular disease.

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