High-Dose Aspirin Is Required to Influence Plasma Fibrin Network Structure in Patients With Type 1 Diabetes

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OBJECTIVE—Patients with type 1 diabetes form a less permeable fibrin network, which could contribute to their increased risk of cardiovascular disease (CVD). Low-dose aspirin treatment is the standard in the management of CVD; however, the effect seems reduced in patients with diabetes. We investigated the effects of low- and high-dose aspirin treatment on fibrin network formation in patients with type 1 diabetes (primary aim) and the possible interaction between the treatment effects of aspirin on fibrin network permeability and glycemic control in these patients (secondary aim).

RESEARCH DESIGN AND METHODS—Forty-eight patients (24 subjects with good [HbA1c <7.4%] and 24 subjects with poor [HbA1c >8.4%] glycemic control) were randomly assigned to treatment with 75 or 320 mg/day aspirin during 4 weeks in a crossover fashion. A 4-week washout period separated the treatment periods. The plasma fibrin network was assessed by determination of the permeability coefficient (Kp).

RESULTS—Treatment with 75 mg aspirin did not influence fibrin network permeability (Kp). However, Kp increased significantly during treatment with 320 mg aspirin (P = 0.004), and a significant treatment effect was seen compared with treatment with 75 mg aspirin (P = 0.009). The increase in Kp during high-dose aspirin treatment was significant in patients with poor glycemic control (P = 0.02), whereas Kp only tended to increase in patients with good glycemic control (P = 0.06).

CONCLUSIONS—A high dose of aspirin is required to influence fibrin network permeability in patients with type 1 diabetes. The observed lack of effect with low-dose aspirin may contribute to aspirin treatment failure in diabetes.

Diabetes is associated with increased platelet activation (1), elevated plasma fibrinogen levels (2), and impaired fibrinolysis (3), factors that may contribute to the elevated risk of cardiovascular disease (CVD) in these patients. Increased platelet activation in patients with diabetes is reflected by elevated levels of platelet microparticles, which are small circulating procoagulant vesicles shed from the platelet membrane upon activation (4). Low-dose aspirin therapy is one of the cornerstones in the management of CVD; however, the preventive effect seems reduced in patients with diabetes (5).

Aspirin inhibits platelet function by irreversibly acetylating a serine residue in cyclooxygenase-1, thereby grossly reducing the production of the platelet-activating and vasoconstrictive compound thromboxane A2. This is the most accepted effect of aspirin in terms of cardiovascular protection. However, aspirin also may influence coagulation through effects on thrombin generation, factor XIII activation, and fibrin network formation (6). The fibrin network is an important part of the arterial thrombus, and its structure may influence the predisposition to atherothrombotic events (7). During thrombin activation, fibrinopeptides are released from fibrinogen, which polymerize and, in the presence of factor XIII, form a cross-linked fibrin network. The structure of the fibrin network is influenced by the environment in which it is formed and affects the fibrinolytic rate (8). A tighter and less permeable fibrin network, which is less susceptible to fibrinolysis, is formed in patients with manifest CVD or conditions associated with increased risk of atherothrombotic complications (7–10). In previous studies, we have shown that patients with type 1 diabetes have reduced fibrin network permeability and that improved metabolic control is associated with increased fibrin network permeability (11,12). The altered fibrin network in patients with type 1 diabetes may in part be attributed to increased fibrinogen glycation, as shown in studies on fibrinogen purified from diabetic patients (13,14). Treatment with aspirin increases fibrin network permeability in nondiabetic subjects, possibly through acetylation of lysine residues on plasma fibrinogen (15–17). However, the effect of aspirin on fibrin network permeability in patients with diabetes is unclear. Possible competition between acetylation and glycation on lysine residues in the fibrinogen molecule might contribute to the reduced preventive effect of aspirin in the management of CVD in patients with diabetes, and higher doses of aspirin might be required in these patients.

The primary aim of the current study was to investigate the effects of low- and high-dose aspirin treatment on fibrin network permeability in patients with type 1 diabetes. The secondary aim was to investigate the possible interaction between the treatment effects of aspirin on fibrin...
network permeability and glycemic control in these patients. Because platelet microparticles may influence the fibrin formation (18,19), and because aspirin has well-known effects on platelet function, we also measured plasma concentrations of platelet microparticles.

RESEARCH DESIGN AND METHODS—A crossover study with randomization to receive a daily dose of 75 or 320 mg aspirin (Trombyl; Pfizer) was designed. The 4-week treatment periods were separated by a 4-week washout period. Investigations were performed at the start and the end of each treatment period. Compliance was checked through tablet counts.

A total of 24 patients (12 women) with good glycemic control (HbA1c <7.4%) and 24 patients (12 women) with poor glycemic control (HbA1c >8.4%) were recruited from the Division of Medicine, Danderyd Hospital, Stockholm, Sweden. Eligible for the study were patients with type 1 diabetes between the ages of 30 and 70 years, without previous aspirin treatment, ongoing nonsteroidal anti-inflammatory or anticoagulant treatment, or a history of macrovascular events. Data on retinopathy status was based on fundoscopic findings. All patients arrived at the laboratory in the morning after a 10-h fast. Clinical signs of peripheral neuropathy were investigated with tests of vibration and superficial sensation using a vibration fork (128 Hz) and monofilament (Semmes-Weinstein 5.07), respectively. Prevalence of albuminuria was assessed by urinary dipstick tests (Clinitek; Bayer HealthCare).

Fibrin network
The fibrin network structure was studied in citrated plasma samples by measurement of the permeability coefficient (Kc), as described in detail elsewhere (20). In brief, plasma samples were dialyzed and supplemented with CaCl2 and thrombin to final concentrations of 20 mmol/L and 0.2 NIH/mL, respectively. The fibrin network permeability coefficient (Kc) was determined following percolation of a TRIS buffer through the formed fibrin clots at five different hydrostatic pressures. Low levels of Kc indicate reduced fibrin network permeability. The interassay coefficient of variation was 9.5%.

Platelet microparticles
Previously frozen platelet poor plasma was thawed and centrifuged at 2,000g for 20 min at room temperature. The supernatant was then recentrifuged at 13,000g for 2 min. Twenty-microliter samples were incubated for 20 min with Phalloidin-Alexa 660 (Invitrogen, Paisley, U.K.), lactadherin–fluorescein isothiocyanate (phosphatedelserine exposure; Hematologic Technologies, Essex Junction, VT), and CD42a-PE (glycoprotein IX; Abcam, Cambridge, U.K.). Microparticles were measured by flow cytometry on a Beckman Gallios instrument (Beckman Coulter, Brea, CA) according to a modified method (21). The microparticle gate was determined using Megamix beads (BioCytex, Marseille, France), which is a mix of 0.5-, 0.9-, and 3-μm diameter beads. Appropriate conjugate isotype-negative control to determine the background noise of the cytometric analysis. The mean concentration of microparticles was calculated as follows: (microparticle counts × standard beads/liter)/standard beads counted (FlowCount; Beckman Coulter).

Biochemical analyses
HbA1c levels were analyzed by the Mono S method using high-performance liquid chromatography (Variant II; Bio-Rad Laboratories, Hercules, CA) and are expressed in National Glycohemoglobin Standardization Program equivalent values. Lipoproteins were assayed enzymatically with reagents from Synchron LX System(s) (Beckman Coulter, Fullerton, CA) and are expressed as mg/dL and percentages of total lipids. C-reactive protein (CRP) was analyzed using immunonephelometry (Dade Behring, Marburg, Germany). Total plasma fibrinogen was analyzed by means of a Fibri-Prest Automate (Abbott Diagnostics Stago).

Statistical analysis
Sample size calculations based on previous results on fibrin network permeability in patients with type 1 diabetes indicated that 14 patients were required in each group (12). In the current study, we included 24 patients in each group to compensate for dropout. Shapiro-Wilk test was used to assess conformity with a normal distribution. Differences between and within the different doses of aspirin treatments and the patients with good and poor glycemic control, respectively, were investigated by means of two-way repeated-measures ANOVA. Data are presented as mean values ± SD, 95% CIs for the mean, the median with lower- to upper-quartile values in parentheses, or as numbers. A probability (P) of <0.05 was considered statistically significant.

Ethical considerations
The protocol of this trial was approved by the local ethics committee of Karolinska Hospital and the Medical Products Agency. Written informed consent was obtained from all patients.

RESULTS—Seven patients did not complete the study (i.e., three patients discontinued because of the possible mild side effects of aspirin, two patients were excluded because of failure of analyzing procedures, one patient was excluded because of poor compliance, and one patient proved not to meet the inclusion criteria). Baseline characteristics of 41 patients who completed the study are summarized in Table 1. There were no significant differences between the patients with good or poor glycemic control regarding mean age, diabetes complications, and antihypertension and statin treatments. The patients with good glycemic control had, compared with the group with poor glycemic control, longer diabetes duration (30 years [19–43] vs. 15 years [10–29], medians with lower-upper quartiles in brackets; P = 0.01), better lipid profile, and lower plasma fibrinogen levels (2.5 ± 0.4 vs. 2.9 ± 0.7 g/L; P = 0.02). No significant differences between the groups

Table 1—Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (women/men)</th>
<th>Age (years)</th>
<th>Diabetes duration (years)</th>
<th>Smokers</th>
<th>BMI (kg/m²)</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Diastolic blood pressure (mmHg)</th>
<th>Antihypertensive treatment</th>
<th>Statin treatment</th>
<th>Proliferative retinopathy</th>
<th>Peripheral neuropathy</th>
<th>Microalbuminuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (women/men)</td>
<td>41 (19/22)</td>
<td>51 ± 12</td>
<td>21 (13–33)</td>
<td>4 (10)</td>
<td>25 ± 3</td>
<td>132 ± 18</td>
<td>75 ± 7</td>
<td>17 (41)</td>
<td>21 (51)</td>
<td>12 (29)</td>
<td>1 (2)</td>
<td>10 (24)</td>
</tr>
<tr>
<td>S-Creatinine (μmol/L)</td>
<td>75 (63–84)</td>
<td>10.1 ± 3.4</td>
<td>2.7 ± 0.7</td>
<td>CRP (mg/L)</td>
<td>1.0 (1.0–2.0)</td>
<td></td>
<td></td>
<td>4.5 ± 0.7</td>
<td>2.5 ± 0.6</td>
<td>1.6 ± 0.4</td>
<td>0.7 (0.5–1.0)</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/L)*</td>
<td>2.7 ± 0.7</td>
<td>1.0 (1.0–2.0)</td>
<td></td>
<td>CRP (mg/L)</td>
<td>1.0 (1.0–2.0)</td>
<td></td>
<td></td>
<td>4.5 ± 0.7</td>
<td>2.5 ± 0.6</td>
<td>1.6 ± 0.4</td>
<td>0.7 (0.5–1.0)</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.7 (0.5–1.0)</td>
<td></td>
<td></td>
<td>CRP (mg/L)</td>
<td>1.0 (1.0–2.0)</td>
<td></td>
<td></td>
<td>4.5 ± 0.7</td>
<td>2.5 ± 0.6</td>
<td>1.6 ± 0.4</td>
<td>0.7 (0.5–1.0)</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD, median (lower to upper quartiles), or n (%). Kc, fibrin network permeability coefficient. *Reference range 2–4 g/L.
with significantly increased fibrin network permeability. Furthermore, we found that the effect of high-dose aspirin on fibrin network permeability was more pronounced in patients with poor metabolic control.

The fibrin network structure seems important in the development of atherothrombotic events, because individuals at high risk of CVD, including patients with type 1 diabetes and nephrotic syndrome, as well as patients with manifest CVD, have a tighter and less permeable fibrin network structure (7,9–11). Our previous studies in patients with type 1 diabetes have shown increased fibrin network permeability during treatments with subcutaneous continuous insulin infusion, statins, and dalteparin, respectively (12,18,22). These studies indicate that fibrin network permeability ($K_s$) is a functional variable suitable for intervention studies.

Aspirin alters the fibrin/fibrinogen properties and thereby influences the fibrin network structure, possibly through acetylation of the lysine residues in the fibrinogen molecule involved in cross-linking of fibrin (15–17). This cyclooxygenase-independent effect of aspirin may add to the antithrombotic effects of aspirin. Increased fibrin network permeability during aspirin treatment has been shown in healthy individuals at daily doses of 37.5, 75, and 320 mg (15,16). In the current study, no effect of low-dose aspirin treatment (75 mg) was found on fibrin network permeability ($K_s$) in patients with type 1 diabetes, whereas treatment with 320 mg aspirin increased $K_s$ levels significantly. Our results thus indicate that patients with type 1 diabetes may require higher doses of aspirin to influence the fibrin network structure. This finding supports the hypothesis that diabetes is associated with a reduced response to aspirin compared with nondiabetic subjects and that higher aspirin doses, rather than the recommended low-dose treatment (defined as 75–162 mg/day, according to the American Diabetes Association), are required in the management of CVD in patients with diabetes.

One plausible explanation behind the observed dose-dependent effect of aspirin on fibrin network permeability in

**CONCLUSIONS**—In the current study, no effect on fibrin network permeability was found during low-dose aspirin (75 mg) treatment in patients with type 1 diabetes, whereas treatment with a high dose of aspirin (320 mg) was associated...
Tabela 2 — Efeitos de 75 e 320 mg de aspirina em pacientes com bom e pior controle glicêmico, respectivamente

<table>
<thead>
<tr>
<th></th>
<th>75 mg aspirina</th>
<th>320 mg aspirina</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alto controle glicêmico (n = 22 [11 masculino])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>2.5 ± 0.6–2.2 ± 0.5</td>
<td>2.6 ± 0.6–2.5 ± 0.5</td>
</tr>
<tr>
<td>Platelet microparticles</td>
<td>24 ± 0.6–23 ± 0.6</td>
<td>26 ± 0.6–25 ± 0.6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.9 ± 0.4–8 ± 0.6</td>
<td>7.0 ± 0.5–8 ± 0.6</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.3 ± 0.4–2.2 ± 0.5</td>
<td>2.7 ± 0.6–2.6 ± 0.7</td>
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</tbody>
</table>

Diabetes é o aumento da glicação de moléculas de fibrinogênio em plasma (13,14). É conhecido que o glicação de fibrinogênio pode ter reduzido a acetilação por aspirina, e competição entre glicação e acetilação de fibrinogênio no grupo amino tem sido observado em in vitro (23). Em suporte a esta hipótese, a interação entre acetilação e glicação no resíduo de linas foi também indicado em estudos sobre albumina plasmática, a glicação rate de linas de resíduo inibido durante incubação simultânea com aspirina e glicose (24). Na atual revisão, no entanto, apenas análises subgrupos analisando a possível influência da glicação sobre os efeitos de aspirina em pacientes com glicemia mais alta dependia da presença de pacientes com pior glicemia. No caso, embora a aspirina possa ser um modificador da resposta do nexo fibrino a ter um impacto significativo no paciente com glicemia mais alta comparado com pacientes com pior glicemia (Tabela 2 e Fig. 2). Entretanto, a aspirina pode não ter um impacto significativo na resposta do nexo fibrino a ter um impacto significativo no paciente com glicemia mais alta comparado com pacientes com pior glicemia (Tabela 2 e Fig. 2).

Plasma fibrinogênio concentração, por outro lado, tem um impacto significativo no nexo fibrino estrutura e, portanto, a nexo fibrino permeabilidade coeficiente (Kₚ) valores em todos os pontos de investigação. Níveis de fibrinogênio são independentes de risco para CVD (25), e níveis elevados são comuns em pacientes com diabetes (2). Em linhas anteriores, encontramos níveis significativamente mais altos de plasma fibrinogênio em pacientes com pior glicemia.

Porque as células micropartículas plaquetárias podem influenciar a permeabilidade do nexo fibrino (18,19), e porque aspirina tem efeitos conhecidos sobre o nexo plaquetário, medimos a concentração plaquetária de plaquetas micropartículas no grupo de controle. No entanto, o efeito de 75 ou 320 mg de aspirina foi medido em pacientes com boa e pior glicemia.

Conclusão — o tratamento com aspirina de alta-dose (320 mg) é necessário para aumentar a permeabilidade do nexo fibrino em pacientes com diabetes tipo 1. O efeito da aspirina com dose baixa pode levar a falha no tratamento antiagregante.


