Clopidogrel Improves Systemic Endothelial Nitric Oxide Bioavailability in Patients With Coronary Artery Disease
Evidence for Antioxidant and Antiinflammatory Effects

Thomas Heitzer, Volker Rudolph, Edzard Schwedhelm, Manuela Karstens, Karsten Sydow, Michelle Ortak, Peter Tschentscher, Thomas Meinertz, Rainer Böger, Stephan Baldus

Background—Platelet stimulation and activation are known not only as prerequisite of clot formation but are increasingly recognized as important contributors to inflammation and vascular injury. The present study in patients with symptomatic coronary disease investigated whether platelet adenosine diphosphate receptor blockade by clopidogrel exerts beneficial effects on endothelial nitric oxide bioavailability, oxidative stress, and/or inflammatory status.

Methods and Results—One hundred three consecutive patients with symptomatic coronary disease and long-term aspirin therapy were studied. Endothelium-dependent and -independent vasodilation was determined measuring forearm blood flow (FBF)-responses to acetylcholine with and without N\textsuperscript{0}-monomethyl-L-arginin (L-NMMA) and sodium nitroprusside, by using venous occlusion plethysmography. Patients were randomized to receive additional treatment with clopidogrel or placebo. Vascular function tests were repeated after 5 weeks and showed significant improvement of acetylcholine-induced vasodilatation and L-NMMA responses in the clopidogrel-added group (max. FBF from 9.8±0.3 to 14.7±0.4; L-NMMA-response from 3.7±0.1 to 6.8±0.3 mL/100 mL/min). In contrast, no significant changes were observed in the placebo group. Sodium nitroprusside-induced vasodilation was not changed in either group. Urinary excretion of 8-iso-prostaglandin F\textsubscript{2}α and plasma levels of hsCRP, sCD40L, and RANTES were reduced in patients on additional treatment with clopidogrel, but not in patients on placebo.

Conclusions—Clopidogrel improves endothelial nitric oxide bioavailability and diminishes biomarkers of oxidant stress and inflammation in patients with symptomatic coronary artery disease, suggesting that beyond inhibition of platelet aggregation, adenosine phosphate receptor blockade may also have promising vasoprotective effects. (Arterioscler Thromb Vasc Biol. 2006;26:1648-1652.)

Key Words: clopidogrel ■ endothelial function ■ nitric oxide ■ platelets ■ coronary artery disease.

A growing body of evidence indicates that platelet stimulation and activation not only induces acute coronary thrombosis but also has unrevealed roles in inflammation and vascular injury.\textsuperscript{1,2} Platelet expression of inflammatory mediators induces endothelial activation and secretion of proinflammatory cytokines resulting in a localized inflammatory response. Interestingly, recent experimental studies demonstrated that inflammation profoundly impairs endothelium-dependent vasodilatation in human microvascular circulation.\textsuperscript{3} In addition, activated platelets are important sources of reactive oxygen species such as superoxide anions known to inactivate endothelium-derived nitric oxide (NO).\textsuperscript{4}

Pharmacological inhibition of platelet glycoprotein (GP) I\textsubscript{IIa}/I\textsubscript{IIa} receptor activation has been shown not only to inhibit platelet aggregation but also to limit the inflammatory response in acute coronary syndromes.\textsuperscript{3} In addition, recent studies demonstrated beneficial effects of GP I\textsubscript{IIa}/I\textsubscript{IIa} inhibitors on endothelial function and NO bioactivity.\textsuperscript{6} Clopidogrel is a member of the thienopyridine group that acts by blocking the platelet adenosine diphosphate (ADP) receptor, and has been shown to be of potential benefit in patients with acute coronary syndromes.\textsuperscript{7} Recent experimental studies suggest that clopidogrel also may have some antiinflammatory effects.\textsuperscript{8,9} However, the influence of clopidogrel on endothelial function has not been investigated yet. Therefore, the primary aim of the study was to determine the effects of additional treatment with clopidogrel on endothelial vasodilator function and NO bioactivity in patients with symptomatic coronary disease on aspirin therapy. The second aim was to further elucidate possible mechanisms of additional treatment with clopidogrel by measuring in vivo parameters of inflammation and oxidative stress.
Methods

Patient Population
Patients who had been referred for evaluation of symptomatic CAD were eligible for the study. Inclusion criteria were assessed as documented, stable CAD and chronic aspirin therapy on a daily dose ≥100 mg/d. CAD was defined as a history of myocardial infarction documented on the basis of enzyme-related criteria, angiographically proven coronary disease (≥50% luminal stenosis), or previous coronary revascularization. Exclusion criteria assessed as unstable angina, acute myocardial infarction, evidence of heart failure, uncontrolled hypertension, oral anticoagulation, bleeding abnormalities, and/or significant endocrine, hepatic, renal or inflammatory disease. Vasoactive medications, including calcium-channel blockers, β-blockers, ACE inhibitors, and long-acting nitrates, were withheld for ≥24 hours before the vascular function study. None of the patients received thienopyridines or glycoprotein IIb/IIIa inhibitors before inclusion into the study. The study was approved by the local ethics committee, and informed consent was obtained from all participants.

Study Protocol
The study was designed in a randomized, placebo-controlled, parallel fashion in which eligible patients underwent randomization in a 3:1 ratio to receive additional platelet inhibition with clopidogrel or matched placebo. Clopidogrel was administered with a loading dose of 300 mg and continued with 75 mg/d. Otherwise, patients were kept on their previous medical treatment including chronic aspirin therapy. A β-blocker or occasional use of nitrates was allowed as additional agents. If not on a lipid-lowering or ACE inhibitor therapy, this medication was withheld during the study period. Patients were followed up in the outpatient clinic. Vascular function tests and collection of blood and urine specimen were done initially, at randomization, and at the 5-week follow-up.

Vascular Function
Vascular function tests were performed after a 12-hour overnight fasting period with the subjects lying supine in a quiet temperature-controlled room. A 20-gauge polyethylene catheter was inserted into the brachial artery of the nondominant arm under sterile conditions for measurement of blood pressure and infusion of drugs. Forearm blood flow (FBF) was measured by venous occlusion plethysmography with calibrated mercury-in-silastic strain gauges as previously described.10 During FBF measurement, blood flow to the hand was prevented by a wrist cuff, inflated 40 mm Hg above systolic blood pressure. At the beginning of each study protocol, saline (0.9% sodium chloride) was infused intravenously at a rate of 0.4 ml/mmin. Endothelium-dependent vasodilatation was assessed by infusing acetylcholine (ACh) in increasing concentrations of 7.5, 15, and 30 μg/min into the brachial artery. Sodium nitroprusside (SNP) was infused to assess endothelium-independent vasodilation (1, 3, and 10 μg/min). The sequence of ACh and SNP infusion was randomized. Then, during coinfusion of the NO-synthase inhibitor Nω-monomethyl-L-arginine (L-NMMA; 16 μmol/min), dose response curves to ACh were assessed.

Laboratory Methods
Routine parameters were obtained on both visits and analyzed on the day of sampling; further samples were separated and the serum/plasma stored at −70°C until analysis. High sensitive C-reactive protein (hsCRP) levels were measured using an ultrasensitive test (CRP Latex HS, Roche Diagnostics). Plasma sCD40L and RANTES were measured by ELISA using commercial kits and reagents (R&D Systems). Plasma NOx concentrations were measured using a colorimetric assay kit that measures total nitrate/nitrite concentration in a 2-step process (Cayman Chemical). Absorbance was measured at 540 nm by a multimode microplate reader (Tecan GENios). Each plasma sample was measured in duplicate. Urinary concentrations of the isoprostane 8-iso-PG_F2α (PGF2α) were determined by gas chromatography mass spectrometry as previously described,41 corrected for urinary creatinine concentrations.

Baseline Characteristics of Study Patients

<table>
<thead>
<tr>
<th></th>
<th>ASA = Placebo (n = 26)</th>
<th>ASA + Clopidogrel (n = 77)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>65 ± 3</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.1 ± 0.6</td>
<td>25.8 ± 0.5</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>12 (46)</td>
<td>34 (44)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>8 (31)</td>
<td>29 (38)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>6 (23)</td>
<td>18 (23)</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>15 (58)</td>
<td>47 (61)</td>
</tr>
<tr>
<td>Multivessel disease, n (%)</td>
<td>10 (39)</td>
<td>36 (47)</td>
</tr>
<tr>
<td>Previous infarction, n (%)</td>
<td>11 (42)</td>
<td>35 (46)</td>
</tr>
<tr>
<td>Forearm volume, L</td>
<td>1.01 ± 0.08</td>
<td>1.10 ± 0.11</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>26 (100)</td>
<td>77 (100)</td>
</tr>
<tr>
<td>Betablocker, n (%)</td>
<td>20 (77)</td>
<td>56 (73)</td>
</tr>
<tr>
<td>ACE-I/ARB, n (%)</td>
<td>16 (62)</td>
<td>46 (60)</td>
</tr>
<tr>
<td>Calcium-Antagonist, n (%)</td>
<td>6 (23)</td>
<td>16 (21)</td>
</tr>
<tr>
<td>Statin, n (%)</td>
<td>20 (77)</td>
<td>57 (74)</td>
</tr>
<tr>
<td>Nitrate, n (%)</td>
<td>4 (15)</td>
<td>11 (14)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM or n (%); BMI indicates body mass index; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin-I receptor antagonist.

Statistical Analysis
The primary end point of the study was the effect of clopidogrel compared with placebo on endothelial function, measured as ACh responses with and without L-NMMA inhibition. Based on previous studies, we calculated the sample size for a power of 90% and a significance level of 0.05. Changes in urinary levels of 8-isoprostaglandin F2α were secondary end points. Data with normal distribution are presented as mean ± SEM and were analyzed by Student paired and unpaired t test. Differences in forearm vascular reactivity were analyzed by repeated-measures ANOVA, and Bonferroni post-hoc test was applied for multiple comparison testing. FBF responses to ACh, SNP, and L-NMMA were calculated as area under the curve and expressed in arbitrary units. Categorical data were compared by the χ² test and the Fisher exact test. Non-normally distributed data, presented as median and interquartile range, were analyzed by the Mann–Whitney U test or Wilcoxon signed rank sum test. A value of P < 0.05 was considered statistically significant.

Results

Patient Characteristics
One hundred thirteen patients with coronary artery disease and chronic aspirin therapy were randomized. During follow-up, 9 patients were excluded because symptoms of angina increased and early coronary angiography and percutaneous coronary intervention had to be performed. One patient withdrew his consent after the initial baseline study. One hundred three patients completed the study; 77 patients were randomized to receive additional treatment with clopidogrel, and 26 patients to placebo. There were no significant differences in baseline characteristics of both groups (Table). Medical treatment apart from antiplatelet medication was comparable and remained unchanged during the study period.

Effects on Vascular Measurements
At baseline, acetylcholine (ACh)-induced increases of forearm blood flow (FBF) were comparable in both groups (Figure 1A and 1B). At follow-up after 5 weeks, patients receiving placebo (ASA + Placebo) did not show any signif-
significant change in ACh-induced vasodilation compared with baseline (Figure 1A). In contrast, patients on adjunct treatment with clopidogrel (ASA+Clopidogrel) revealed significant improvement of ACh-induced vasodilation (Figure 1B). At baseline, inhibition of NO synthesis by infusion of L-NMMA reduced ACh-induced FBF in both groups to a comparable amount (Figure 2A and 2B). At follow-up, the inhibitory effect of L-NMMA was unchanged in patients on ASA+Placebo (AUC 77±18 versus 84±16) (Figure 2A). However, this L-NMMA effect was significantly increased in patients on ASA+Clopidogrel compared with baseline (AUC 72±12 versus 169±16; Figure 2B). Endothelium-independent vasodilation in response to sodium nitroprusside was not altered in either group (Figure 3).

Effects on Biochemical Parameters

At baseline, urinary excretion of 8-iso-PGF$_{2\alpha}$ was comparable in both groups. At follow-up, however, excretion of 8-iso-PGF$_{2\alpha}$ was significantly reduced in the group on ASA+Clopidogrel, whereas excretion was not altered in the ASA+Placebo group (Figure 4).

Plasma parameters at baseline and at follow-up are shown in Figure 5. Plasma levels of sCD40L were comparable at baseline. After 5 weeks treatment, plasma sCD40L levels declined in patients on ASA+Clopidogrel, as opposed to patients on ASA+Placebo. Plasma concentrations of RANTES did not differ between both groups at baseline. After 5 weeks, however, RANTES concentrations declined in patients on ASA+Clopidogrel but were unchanged in the group with ASA+Placebo. Plasma concentrations of hsCRP were comparable in both groups at baseline. At follow-up, hsCRP was significantly reduced in patients on ASA+Clopidogrel, but not in the group on ASA+Placebo. NOx plasma concentrations were almost identical in both groups at baseline, but increased significantly in patients on ASA+Clopidogrel at follow-up while remaining unaltered in the group on ASA+Placebo.

Discussion

The present study demonstrates that platelet ADP receptor blockade by clopidogrel exerts beneficial effects on systemic nitric oxide bioavailability in patients with coronary artery disease, supporting the concept that abnormal platelet–endothelial interactions contribute to endothelial dysfunction. Clopi-
Reduced systemic NO bioavailability. At follow-up after 5 weeks, both ACh-induced vasodilation and L-NMMA-induced reduction of FBF were significantly improved in patients on additional treatment with clopidogrel, but remained unchanged in patients on aspirin monotherapy. Because endothelium-independent vasodilation to SNP did not change in either group, this finding strongly suggests that platelet ADP receptor blockade protects against dysfunctional platelet–endothelial interactions rather than a nonspecific interaction with the vascular wall.

Traditionally, platelets have been recognized as anuclear small discs circulating in the blood and primarily involved in regulating thrombosis and hemostasis. Recently, the anuclear platelet is being rediscovered as an intriguing link between thrombosis and inflammation, and several studies provided insights into platelet-dependent mechanisms leading to endothelial activation and lesion progression. Stimulated platelets are known to be important sources of chemokines and cytokines, serving as reservoirs for proinflammatory activity and able to initiate inflammatory responses on endothelial cells. One of the most important inflammatory platelet mediators is CD40L. Increased CD40L concentrations have been found in patients with symptomatic CAD. Previous studies demonstrated that GP IIb/IIIa receptor blockade has an inhibitory effect on the release of CD40L. Similarly, clopidogrel completely abolishes ADP-induced expression of platelet surface CD40L via blockade of the P2Y12 ADP receptor. In the present study, we found a significant reduction of plasma levels of sCD40L after treatment with Clopidogrel, a finding consistent with other recently published studies. Furthermore, activated platelets express and secrete the proinflammatory chemokine RANTES, which has been shown to trigger enhanced recruitment of monocytes on inflamed/activated endothelium. In our study, Clopidogrel also reduced hsCRP levels, a sensitive systemic marker of inflammation and known to be an important prognostic predictor for coronary risk. Interestingly, recent studies in patients with CAD revealed a strong association between proinflammatory and anti-inflammatory imbalance (i.e., hsCRP and IL-10 serum levels) and impaired systemic endothelial function and NO bioavailability. Our findings that reduction of hsCRP levels over time was associated with a significant improvement in endothelial function are in line with these studies.

Enhanced production of oxygen-derived free radicals leads to impaired NO bioavailability, increased platelet activation, and abnormal platelet–endothelial interactions. To assess oxidative stress status in vivo, quantification of F2-isoprostanes has been shown to be a useful and well established biomarker. In the present study, we observed a significant reduction of 8-iso-PG F₂α in patients after additional treatment with clopidogrel, whereas no changes were seen in patients on aspirin monotherapy. This selective improvement in a relatively small population is remarkable and suggests...
that reducing oxidative stress may be an important mechanism by which clopidogrel exerts its beneficial effects on NO bioavailability. It is well known that platelets themselves represent a powerful source of reactive oxygen species (ROS), and ROS released in response to platelet homotypic and/or heterotypic interactions with leukocytes may decrease vascular NO bioavailability leading to impaired endothelial function. Of note, it has recently been shown that CD40L reduces endothelial NO bioavailability by increasing endothelial production of ROS. Because clopidogrel has an inhibitory effect on the release and interactions of CD40L, this may represent an additional important mechanism by which clopidogrel exerts beneficial effects on endothelial NO bioavailability. Interestingly, a recent study demonstrated that glycoprotein IIb/IIIa inhibition both decreases platelet superoxide release and enhances platelet NO production. Whether clopidogrel has similar intrinsic activating properties remains to be investigated. Our finding of increased NOx plasma levels in patients after chronic clopidogrel treatment is well in line with the observed reduced oxidative stress and improved systemic endothelial NO bioavailability.

In summary, in patients with symptomatic CAD, treatment with clopidogrel improved systemic endothelial function and NO bioavailability, reduced oxidative stress, and decreased the inflammatory response. These findings support the tenet that beyond its antiaggregatory effect, platelet ADP receptor blockade may represent an important agent for the inflamed “vulnerable” patient with systemic inflammatory activation, increased oxidative stress burden, and endothelial dysfunction. Thus, in the clinical setting of symptomatic patients prone to acute vascular injury, clopidogrel may reduce vascular events not only by preventing acute thrombosis but also by exerting its beneficial vascular effects.

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Disclosures
None.

References
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