Cardiovascular thrombotic events facilitated by preexisting atherosclerotic lesions account for about two thirds of deaths in diabetic patients. In addition to macrovascular events, activated platelets contribute to microvascular occlusion, embolization of platelet–platelet or platelet–leukocyte aggregates, and amplification of athero- and thrombogenesis.1

Platelet activation occurs in several cardiovascular diseases with reduced NO bioavailability such as acute coronary syndrome,2,3 heart failure,4–6 insulin resistance,7 and diabetes.8,9 Platelet activation leads to shape change, degranulation, and rapid surface-expression of adhesion molecules such as P-selectin and CD40-ligand.10 P-selectin participates in platelet–platelet or platelet–leukocyte aggregates, and amplification of athero- and thrombogenesis.1

Objective—Platelet activation significantly contributes to cardiovascular morbidity and mortality in diabetes. An association between impaired NO-mediated platelet inhibition and platelet activation has recently been demonstrated in experimental diabetes. Guanylyl cyclase activation enhances the reduced signaling via the NO/cGMP pathway. We investigated whether chronic guanylyl cyclase activation would beneficially modulate platelet activation in experimental diabetes mellitus.

Methods and Results—Diabetes was induced by streptozotocin-injection in male Wistar rats. After 2 weeks, treatment with either placebo or the guanylyl cyclase activator HMR1766 (10 mg/kg twice daily by gavage) was initiated. Two weeks later, in vivo platelet activation and in vitro platelet reactivity were assessed. Chronic treatment with HMR1766 enhanced NO/cGMP-mediated signaling in platelets from diabetic rats determined by in vivo phosphorylation of platelet vasodilator-stimulated phosphoprotein (VASP) at Ser157 and Ser239. In parallel, platelet-binding of fibrinogen, surface-expression of P-selectin, appearance of platelet-derived microparticles, and platelet-aggregates with other blood cells were significantly reduced by chronic treatment with HMR1766.

Conclusion—Chronic activation of soluble guanylyl cyclase in diabetic rats improved markers of platelet activation and is a rationale approach for prevention of adverse cardiovascular events in diabetes. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: guanylyl cyclase ■ platelet activation ■ diabetes

We recently demonstrated that acute18 and chronic19 reduction of systemic NO bioavailability results in platelet activation in vivo. In addition to its effects on vascular tone, NO is a central regulator of platelet activation, adhesion, and aggregation: reduced NO bioactivity is associated with arterial thrombosis in animal models and in individuals with endothelial dysfunction.20 Recently, platelet NO responsiveness has been found to be a prognostic marker in acute coronary syndromes.21 Functional uncoupling of endothelial NO synthase (eNOS) critically contributes to reduced NO bioavailability in diabetes and increased vascular superoxide generation in diabetes.22 Increased expression of NAD(P)H oxidase subunits, enhanced NAD(P)H oxidase, and protein kinase C activity as well as increased levels of the endogenous eNOS inhibitor asymmetrical dimethylarginine result in enhanced oxidative stress and reduced NO bioavailability in diabetes.23 We recently demonstrated that normalization of vascular NO formation reduces platelet activation by an NO/cGMP-mediated signaling pathway.19 These results underline the critical role of systemic NO bioavailability for regulation/inhibition of platelet activation.

NO/cGMP-dependent phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) plays a pivotal inhibitory
role in the regulation of platelet activation.24 Phosphorylation of VASP correlates closely with inhibition of fibrinogen binding to the platelet glycoprotein (GP) IIb/IIIa receptor.25,26 Increased NO bioavailability induces VASP phosphorylation preferentially at its serine residues 239 (Ser239) and 157 (Ser157) by NO-dependent activation of soluble guanylyl cyclase (sGC) and subsequent cGMP-mediated stimulation of cGMP-dependent kinases (cGK).24,27 By modulating platelet actin filament interactions, phosphorylation of VASP is able to affect initial sequences in platelet adhesion and activation.28–31 cGK, as well as VASP-deficient mice, display enhanced platelet adhesion in vivo in the absence of any proaggregatory stimuli, further strengthening the relevance of endogenous platelet inhibition through the NO/cGMP pathway.28,29

In the present study, we examined the effects of chronic sGC activation on platelet activation in diabetes using the novel direct sGC activator HMR1766.32 We found that sGC activation enhanced the NO/cGMP-mediated platelet inhibitory pathway, thereby reduced in vivo platelet activation and decreased in vitro platelet reactivity.

Materials and Methods

The investigation conforms with the position of the American Heart Association (AHA) on research animal use adopted by AHA on November 11, 1984.

Animals

Male Wistar rats (250 to 300 g; obtained from Harlan-Winkelmann, Borchen, Germany) were housed in temperature-controlled cages (20 to 22°C) with a 12-hour light-dark cycle, and given free access to water and formulated diets.

Induction of Diabetes by Streptozotocin Injection

A single dose streptozotocin (STZ) regimen was used to induce pancreatic islet cell destruction and persistent hyperglycaemia. STZ (10 mg/mL; Sigma) was freshly dissolved in sterile sodium citrate buffer (25 mmol/L, pH 4.5) and used within 10 minutes. Rats received a single 50 mg/kg intravenous injection of STZ. Blood glucose was monitored using a one-touch blood glucose meter (Ascensea Elite; Bayer-Vital GmbH). Hyperglycaemia was defined as the CD42 expression being outside any defined cell population. PMP were investigated in control and diabetic rats in separate experiments 2 hours after application of the study drug by gavage.

Platelet Sampling

General anesthesia was induced using isoflurane. The abdominal cavity was opened under deep anesthesia, determined by total absence of reaction to pain under spontaneous respiration, and blood was taken by direct puncture of the inferior caval vein into a chilled tube containing 3.8% sodium citrate.

Flow Cytometry

Whole blood was diluted with PBS (free of Ca2+ and Mg2+, enriched with D-Glucose [5.5 mmol/L] and 0.5% BSA). Platelet-bound fibrinogen was determined by incubation with a fluorescein isothiocyanate (FITC)-labeled anti-fibrinogen antibody (WAK-Chemie) for 10 minutes. For determination of surface-expressed P-selectin, diluted blood was incubated with a polyclonal rabbit anti-P-selectin (CD62P) antibody (Becton Dickinson) for 10 minutes at room temperature followed by incubation with a FITC-labeled goat anti-rabbit IgG-antibody (Jackson ImmunoResearch). Staining of the samples was also performed only with the FITC-conjugated secondary antibody in the absence of the primary antibody. An anti-rat CD42 monoclonal FITC-conjugated antibody (Becton Dickinson) was used as a platelet-specific marker for detection of circulating platelet derived microparticles (PMP) and platelet aggregates with non-platelet blood cells. Platelet CD42 expression was not significantly different between the two diabetic groups, but differed significantly between diabetic and controls, which did not allow the inclusion of a control group for the above-mentioned parameters. After incubation with the antibodies, platelets were fixed with methanol-free formaldehyde (1.5%) for 10 minutes, and subsequently analyzed in a Becton Dickinson FACSCalibur at a low flow rate. The platelet population was identified on its forward and side scatter distribution, and 20,000 events were analyzed for mean fluorescence using CELLQuest software, version 3.1f; unspecific binding was arbitrarily adjusted to a mean fluorescence of 10 and visually subtracted in the graphs.

The phosphorylation of VASP was evaluated using FITC-labeled antibodies against phosphorylation of Ser239 (16C2 antibody [500 μg/mL]; Nano Tools) and Ser157 (5C6 antibody [500 μg/mL]; Nano Tools).2,27 after fixation of the blood samples by methanol-free formaldehyde (1.5%, 5 minutes). The samples were diluted with PBS and were allowed to permeabilize for 10 minutes after Triton X 100 (0.2% final) had been added. Samples were divided into two, and one portion was stained at room temperature for 45 minutes with the respective FITC-labeled antibody (16C2 or 5C6), the other with the FITC-labeled antibody, which had been preincubulated with a saturating dose of a specific blocking phospho-peptide (incubation lasted at least 30 minutes at 4°C) to control for nonspecific binding, which was arbitrarily adjusted to a mean fluorescence of 10. Phosphorylation of VASP was determined by flow cytometry with a platelet-specific marker for detection of circulating platelet derived microparticles (PMP) and platelet aggregates with non-platelet blood cells, the leukocyte/erythrocyte population was characterized by its typical forward-sideward scatter distribution, 20,000 events within this region were counted and analyzed for CD42 events. For detection of PMP, all events in a whole blood sample were acquired until 20,000 events had been counted within a platelet gate. Microparticles were characterized by forward- and sideward-scatter of <30 and being outside any defined cell population. PMP were defined as the CD42+ events within this microparticle region and the amount of PMP was expressed as CD42+ microparticles/all CD42+ events.

Platelet In Vitro Stimulation

Whole blood was stimulated with ADP (10 μmol/L) before antibody incubation, and PMP formation was assessed as described above. For assessment of in vitro-stimulated P-selectin surface expression, platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood at 180g for 12 minutes at room temperature, after which PRP was stimulated with ADP (5 μmol/L and 10 μmol/L). VASP phosphorylation was measured after PRP had been stimulated with HMR1766 (10 μmol/L) or DEA-NOOdate (1 μmol/L) or both 10 minutes before fixation.

Platelet Aggregation

Platelet aggregation was induced by different concentrations of ADP in PRP using a commercial platelet aggregation profiler (PAP-8; BioData). PRP from untreated STZ diabetic rats, PRP from STZ diabetic rats receiving one single administration of HMR1766 in vivo by gavage 2 hours before blood sampling, and PRP from STZ diabetic rats incubated with HMR1766 in vitro 5 minutes before induction of platelet aggregation by ADP was tested to determine acute effects of HMR1766 on platelet reactivity.

Substances

Unless stated otherwise, all chemicals were obtained from Sigma in the highest purity available.
Table 1. Metabolic and Hematological Parameters From Non-Diabetic Control Rats and Diabetic Rats Treated Either With Placebo or the sGC Activator HMR1766

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>STZ Placebo</th>
<th>STZ HMR 1766</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mg/dL</td>
<td>147±5.8</td>
<td>518±9**</td>
<td>502±11**</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>354±5.6</td>
<td>244±4**</td>
<td>245±5**</td>
</tr>
<tr>
<td>Platelets, n*1000/µL</td>
<td>673±35</td>
<td>599±43</td>
<td>570±34</td>
</tr>
<tr>
<td>Leukocytes, n*1000/µL</td>
<td>5.6±0.5</td>
<td>5.1±0.5</td>
<td>4.8±0.3</td>
</tr>
<tr>
<td>Mean platelet volume, fl</td>
<td>6.02±0.09</td>
<td>6.23±0.09*</td>
<td>5.97±0.0444</td>
</tr>
<tr>
<td>Spleen volume, µL/ g BW</td>
<td>2.01±0.07</td>
<td>1.88±0.05</td>
<td>1.93±0.06</td>
</tr>
<tr>
<td>INR</td>
<td>0.80±0.01</td>
<td>0.78±0.01</td>
<td>0.80±0.01</td>
</tr>
<tr>
<td>PTT, sec</td>
<td>27.3±1.9</td>
<td>34.8±1.2</td>
<td>32.1±1.4</td>
</tr>
</tbody>
</table>

*P<0.05; **P<0.01 vs Control; ***P<0.01 vs STZ-Placebo.

Results

Blood glucose levels, body weight, platelet and leukocyte counts as well as coagulation parameters did not differ between the groups. However, mean platelet volume (MPV), which has been described as a surrogate marker for platelet activation,35 was significantly reduced by chronic treatment with HMR1766 (Table).

Platelet VASP Phosphorylation

The NO/cGMP-axis in platelets was determined using the phosphorylation state of platelet VASP. In vitro stimulation of PRP with the NO donor DEA-NONOate resulted in significant VASP phosphorylation in platelets from control rats, whereas the response was significantly attenuated in platelets from diabetic rats. Incubation of PRP with HMR1766 (10 µmol/L) stimulated VASP phosphorylation more effectively than DEA-NONOate (1 µmol/L). NO sensitivity in platelets from diabetic rats was not significantly enhanced by HMR1766 in vitro (Figure 1A). 2 hours after single enteral application of HMR1766 (10 mg/kg) in vivo, NO sensitivity in platelets from diabetic rats was comparable to the sensitivity seen in platelets from untreated control rats (Figure 1B).

To determine the integrity/activity of the NO/cGMP-signaling pathway after chronic treatment with HMR1766, we assessed the basal phosphorylation state of platelet VASP in whole blood, which was immediately fixed in formaldehyde after collection. Basal platelet VASP phosphorylation at Ser157 (Figure 1C) and Ser239 (Figure 1D) was significantly reduced in diabetic versus control rats and significantly improved in diabetic rats chronically treated with the sGC activator HMR1766 indicating increased cGMP-mediated signaling following chronic activation of sGC.

Platelet Activation

The extent of in vivo platelet activation was measured by analysis of platelet-bound fibrinogen reflecting glycoprotein Ib/IIa activation (Figure 2A) and surface expression of P-selectin as a marker of platelet degranulation (CD62P, Figure 2B) in unstimulated whole blood. Platelet-bound fibrinogen and surface-expressed P-selectin were both significantly reduced by chronic sGC activation with HMR1766. Typical flow cytometry histograms show the leftward shift in fibrinogen-binding (Figure 2C) and P-selectin surface-expression (Figure 2D) by HMR1766 indicating reduced activation.

The amount of circulating platelet aggregates with non-platelet blood cells, determined as the CD42+ fraction of leukocytes/erythrocytes, was also significantly reduced by chronic sGC activation with HMR1766 (Figure 3A) as was the amount of platelet-derived microparticles in whole blood (Figure 3B).

Platelet In Vitro Stimulation

ADP-induced platelet aggregation in PRP from diabetic rats receiving one single dose of HMR1766 (10 mg/kg) in vivo by

Figure 1. Platelet VASP phosphorylation after in vitro stimulation platelet-rich plasma from diabetic (STZ) and non-diabetic rats (control) with HMR1766 (10 µmol/L) or DEA-NONOate (1 µmol/L) or both (A). Effect of 2 hours in vivo treatment with HMR1766 (10 mg/kg) on NO sensitivity in platelets from diabetic vs non-diabetic rats (B). Results are expressed in % of unstimulated VASP phosphorylation, mean±SEM from 5 to 7 animals, *P<0.05 vs control and STZ-HMR1766. Basal VASP phosphorylation at Ser157 (C) and Ser239 (D) in platelets from healthy control and diabetic rats treated either with placebo or the sGC activator HMR1766 for 2 weeks. Results are expressed as the mean fluorescence±SEM from 10 to 16 separate animals, *P<0.05 vs control and STZ-HMR1766.
gavage 2 hours before blood sampling was significantly attenuated compared with PRP from untreated diabetic rats. Incubation of diabetic PRP with HMR1766 (10 μmol/L) in vitro 5 minutes before induction of platelet aggregation by ADP resulted in nearly complete suppression of platelet aggregation (Figure 4A).

Chronic treatment with HMR1766 significantly reduced ADP (10 μmol/L)-stimulated surface expression of P-selectin in PRP (Figure 4B). Similarly, in vitro formation of platelet-derived microparticles in whole blood after ADP-induced (10 μmol/L) stimulation was significantly attenuated in HMR1766-treated diabetic rats (Figure 4C).

**Discussion**

The present study demonstrates that chronic treatment of diabetic rats with the sGC activator HMR1766 enhances signaling through the endogenous platelet-inhibiting NO/cGMP pathway and reduces platelet activation in vivo.

Reduced NO bioavailability in diabetes is mainly attributed to vascular endothelial dysfunction leading to attenuated signal transduction through the endogenous platelet-inhibiting NO/cGMP pathway. The resulting higher susceptibility of platelets to activating factors may be relevant for platelet activation in diabetes. We previously demonstrated that acute inhibition of NO/cGMP signaling rapidly activates circulating platelets in healthy human volunteers and in rodents. Permanent interruption of this endogenous platelet-inhibiting pathway causes enhanced adhesion of circulating platelets on intact endothelium. Pharmacological sGC activation exerts antiplatelet properties in vitro by increasing platelet cGMP, the main mediator of NO effects in platelets. We demonstrate here that acute application of HMR1766 improves platelet signaling through the NO/cGMP/VASP pathway and reduces platelet reactivity. The results from in vitro and acute in vivo experiments suggest that the stimulation of sGC by HMR1766 is independent of sGC dysfunction in diabetic rats. Furthermore, the present study provides the first in vivo evidence...
that chronic sGC stimulation enhances the endogenous platelet-inhibiting NO/cGMP pathway as demonstrated by the increased VASP phosphorylation, and reduces platelet activation in diabetic rats. Chronic sGC stimulation in vivo attenuated the activation of glycoprotein Ib/IIa (determined by platelet fibrinogen-binding) as well as platelet degranulation (determined by P-selectin surface expression). P-selectin can participate in platelet adhesion to the endothelium and is responsible for platelet-leukocyte adhesion.\textsuperscript{11,12} P-selectin-expressing platelets play a pivotal role in the interaction of activated platelets with leukocytes and for exacerbation of atherosclerosis.\textsuperscript{16} Earlier studies reported inhibition of P-selectin and CD40-ligand surface expression by cAMP/cGMP-dependent protein kinases,\textsuperscript{10} whose activation can be monitored by VASP-phosphorylation.\textsuperscript{27} Consequently, chronic sGC activation by HMR1766 reduced platelet aggregation to non-platelet blood cells and decreased the amount of circulating platelet-derived microparticles. In vitro application of HMR1766 did increase sGC activity, but did not enhance NO sensitivity. In contrast, in vivo treatment with HMR1766 did enhance platelet sensitivity to exogenous NO. Interestingly, a similar pattern was observed in isolated aortic rings from these animals, in which HMR1766 improved vasorelaxation to exogenous NO and reduced oxidative stress.\textsuperscript{39} Impaired platelet NO responsiveness is a predictor of increased mortality and cardiovascular morbidity in patients with acute coronary syndromes.\textsuperscript{71} Improved NO responsiveness by long-term treatment with an sGC activator in diabetes may reduce excess adverse cardiovascular events.

Patients with diabetes have an increased risk of thrombosis and accelerated atherogenesis. Platelet degranulation in patients with diabetes is associated with progression of proatherosclerotic vessel wall modification.\textsuperscript{40} Chronic activation of sGC by HMR1766 in diabetic rats reduced platelet susceptibility to ADP. Increasing levels of glucose have been identified as independent predictors of platelet-dependent thrombosis in patients with coronary artery disease.\textsuperscript{41} Furthermore, markers of platelet activation were already significantly increased in individuals positive for islet cell antibodies before onset of overt diabetes mellitus, indicating that platelet activation occurs very early during the development of diabetes.\textsuperscript{42} This is clinically reflected by the fact that patients with type 2 diabetes without prior cardiovascular events have a risk of myocardial infarction similar to that among nondiabetic patients with prior myocardial infarction.\textsuperscript{43} Thus, activated platelets have a major impact on morbidity and mortality, as most diabetic patients die from cardiovascular atherothrombotic events.\textsuperscript{44} Platelet activation has recently been shown to be one of the initial steps during the development of atherosclerosis,\textsuperscript{16,17} and inhibition of platelet activation/adhesion prevented leukocyte adhesion and plaque development.\textsuperscript{17} Platelets from diabetic patients are characterized by a variety of abnormalities, including increased MPV, higher expression of adhesion molecules on the platelet surface, as well as increased release of vasoconstrictors. The etiology of these abnormalities is attributed to several changes in the diabetic environment; endothelial dysfunction contributing to less endogenous platelet inhibition and increased sensitivity to platelet agonists, oxidative stress, and advanced glycation end products, which either directly influence metabolism in platelets or worsen structural changes in the vessel wall; and inflammation (as recently reviewed).\textsuperscript{45} Hence, enhancement of NO-mediated platelet inhibition by chronic sGC activation in this study resulted in reduced MPV, less fibrinogen binding on GP Ib/IIa, and lower platelet surface expression of the adhesion molecule P-selectin suggesting that loss of chronic NO-mediated platelet inhibition predisposes and contributes to the exaggerated platelet susceptibility observed in diabetes.

**Conclusion**

Enhancement of the endogenous platelet-inhibiting NO/cGMP pathway by chronic treatment with the direct sGC activator HMR1766 reduced platelet activation in diabetic rats. Positive modulation of the endogenous platelet inhibi-
ing pathway is thereby a worthwhile therapeutical option to prevent atherothrombotic complication in diabetes.

Acknowledgments

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Disclosures

None.

References

Soluble Guanylyl Cyclase Activation With HMR1766 Attenuates Platelet Activation in Diabetic Rats
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