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

Andreas Schäfer, Ulrike Flierl, Anna Kobsar, Martin Eigenthaler, Georg Ertl, Johann Bauersachs

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

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

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

Original received March 16, 2006; final version accepted September 21, 2006.
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Arterioscler Thromb Vasc Biol is available at http://www.atvbaha.org
DOI: 10.1161/01.ATV.0000249407.92147.12

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role in the regulation of platelet activation. Phosphorylation of VASP correlates closely with inhibition of fibrinogen binding to the platelet glycoprotein (GP) IIb/IIIa receptor. 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). By modulating platelet actin filament interactions, phosphorylation of VASP is able to affect initial sequences in platelet adhesion and activation. 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.

In the present study, we examined the effects of chronic sGC activation on platelet activation in diabetes using the novel direct sGC activator HMR1766. 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, Borchcn, 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 (Ascensia Elite; Bayer-Vital GmbH). Hyperglycaemia was defined as random blood glucose level >20 mmol/L at 2 and 4 weeks after injection. Diabetic rats were randomized to placebo or the direct sGC activator HMR1766 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 preincubated 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.

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.
Platelets, n*1000/H11006

4** 245

Body weight, g 354

5.6 244

/5.8 518

Blood glucose, mg/dL 147

INR 0.80

PTT, sec 27.3

HMR1766 (10

platelets from diabetic rats. Incubation of PRP with

rats, whereas the response was significantly attenuated in

significant VASP phosphorylation in platelets from control

of PRP with the NO donor DEA-NONOate resulted in

phosphorylation state of platelet VASP. In vitro stimulation

The NO/cGMP-axis in platelets was determined using the

Platelet VASP Phosphorylation

activation,35 was significantly reduced by chronic treatment

between the groups. However, mean platelet volume (MPV),
counts as well as coagulation parameters did not differ

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 sensitiv-
ity in platelets from diabetic rats was not significantly
enhanced by HMR1766 in vitro (Figure 1A). 2 hours after
one 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 un-
treated 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 formalde-
hyde 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
IIb/IIIa 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 signif-
icantly 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
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

Statistics

Values are means±SEM. Statistical analysis was performed by
unpaired two-sided Student t test or one-way ANOVA where
appropriate; platelet reactivity to different doses of ADP (Figure 4B)
was assessed using a Kruskal–Wallis test; P<0.05 was considered
statistically significant.

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

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 treat-
ment with HMR1766 (10 mg/kg) on NO
sensitivity in platelets from diabetic vs
non-diabetic rats (B). Results 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 fluores-
cence±SEM from 10 to 16 separate ani-
mals, **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. Preservation of endogenous platelet inhibition through NO/cGMP signaling in early diabetes prevents from platelet activation in diabetic mice. However, recent data suggest a significant uncoupling of eNOS in diabetes resulting in eNOS mediated superoxide generation. Therefore, besides strategies like enhancing eNOS expression or increasing its activity, another strategy using a direct sGC activator such as the novel heme-oxidized sGC activating anthranilic acid derivative HMR1766 is desirable. 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. P-selectin-expressing platelets play a pivotal role in the interaction of activated platelets with leukocytes and for exacerbation of atherosclerosis. Earlier studies reported inhibition of P-selectin and CD40-ligand surface expression by cAMP/cGMP-dependent protein kinases, whose activation can be monitored by VASP-phosphorylation. 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. Impaired platelet NO responsiveness is a predictor of increased mortality and cardiovascular morbidity in patients with acute coronary syndromes. Improved NO responsiveness by chronic treatment with an sGC activator in diabetes may reduce ex vivo platelet reactivity.

Patients with diabetes have a decreased risk of thrombosis and accelerated atherogenesis. Platelet degranulation in patients with diabetes is associated with progression of proatherosclerotic vessel wall modification. 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. Additionally, 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. 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. Thus, activated platelets have a major impact on morbidity and mortality, as most diabetic patients die from cardiovascular atherothrombotic events. Platelet activation has recently been shown to be one of the initial steps during the development of atherosclerosis, and inhibition of platelet activation/adhesion prevented leukocyte adhesion and plaque development. 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). 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 inhibit-
ing pathway is thereby a worthwhile therapeutic option to prevent atherothrombotic complication in diabetes.

Acknowledgments
The authors thank Meike Leutke, Christian Vogt, and Melinda Hemberger for expert technical assistance.

Source of Funding
This study was partly supported by a research grant from Sanofi-Aventis.

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. published online October 5, 2006;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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