Rosuvastatin Reduces Platelet Activation in Heart Failure
Role of NO Bioavailability

Andreas Schäfer; Daniela Fraccarollo, Martin Eigenthaler, Piet Tas, Andreas Firnschild, Stefan Frantz, Georg Ertl, Johann Bauersachs

Objectives—Endothelial dysfunction and platelet activation are part of the cardiovascular phenotype in congestive heart failure (CHF). We investigated whether 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibition would beneficially modulate vascular NO bioavailability and platelet activation in experimental CHF.

Methods and Results—Chronic myocardial infarction was induced by coronary ligation in male Wistar rats. Animals were either treated with placebo or the HMG-CoA reductase inhibitor rosuvastatin. After 10 weeks, hemodynamic assessment was performed and endothelial function was determined in organ bath studies. NO bioavailability was assessed by in vivo platelet vasodilator-stimulated phosphoprotein (VASP) phosphorylation. Markers of platelet degranulation (surface expression of P-selectin and glycoprotein 53) were determined as well as the amount of circulating platelet–leukocyte aggregates. Endothelium-dependent, acetylcholine-induced vasorelaxation was significantly impaired in aortic rings from CHF rats and improved by rosuvastatin. In parallel, in vivo VASP phosphorylation reflecting NO bioavailability was significantly attenuated in platelets from CHF rats and normalized by rosuvastatin. Platelet activation, which was increased in CHF, was reduced by treatment with rosuvastatin.

Conclusion—HMG-CoA reductase inhibition improved endothelial function, increased systemic NO bioavailability and inhibited exaggerated platelet activation in CHF rats. These mechanisms may contribute to the beneficial effects of statin treatment in CHF. (Arterioscler Thromb Vasc Biol. 2005;25:1071-1077.)

Key Words: endothelial dysfunction ■ nitric oxide ■ platelet activation ■ CHF ■ HMG-CoA reductase inhibition

The endothelium plays a crucial role in the control of vascular tone by releasing endothelium-derived autacoids, the most important of which is NO, generated by endothelial NO synthase (eNOS).1 Endothelial dysfunction contributes to the development of impaired coronary and systemic perfusion as well as reduced exercise capacity in patients with congestive heart failure (CHF). Reduced NO bioavailability and abundant formation of reactive oxygen species (ROS) within the vascular wall are the key determinants in endothelial dysfunction resulting in an imbalance between NO and ROS.2

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.3 We demonstrated recently that loss of systemic NO bioavailability rapidly4 and chronically5 increased platelet activation. The finding that platelets adhere to dysfunctional endothelium and that expression of potential adhesion molecules is enhanced under these conditions suggests that endothelium-derived NO modulates platelet activation in vascular disease states.6

Increased platelet activation is also observed in CHF7-8 and is likely to contribute to cerebral or peripheral thromboembolic events.9 An increased incidence of circulating platelet aggregates has been observed in CHF patients compared with healthy controls.10 This is reflected by the increase of surface-expressed P-selectin on platelets in CHF patients.11 We reported increased platelet activation in an experimental model of severe heart failure in rats. Complete inhibition of the renin-angiotensin-aldosterone system resulted in normalization of endothelial function12 and prevented platelet activation.8

Two very recently published observational studies report improved survival in CHF patients taking statins.13,14 Experimental data support a potent modulatory effect of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibition on sympathetic tone and autonomic function in CHF.15 In addition, pleiotropic effects of statins in CHF include anti-inflammatory features, reduction of ROS, and improvement of NO bioavailability.16-18 Rosuvastatin increased vascular endothelial NO production and attenuated myocardial necrosis after ischemia and reperfusion in mice.19 However,
the influence of statin treatment on platelet activation in CHF is unknown.

Therefore, we examined the therapeutic effects of HMG-CoA reductase inhibition with rosuvastatin on ROS formation, endothelial vasomotor function, and platelet activation in CHF rats after experimental myocardial infarction (MI). We hypothesized that HMG-CoA reductase inhibition would improve endothelial function, enhance NO bioavailability, and reduce platelet activation in CHF.

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 and Induction of CHF

To obtain animals with severe CHF, we used the experimental model of chronic MI in the rat. Rats were randomized to placebo or rosuvastatin (AstraZeneca) 20 mg/kg per day by oral gavage, which is the 90% HMG-CoA reductase–inhibiting dose in rats. After 2 days of treatment, Sham operation or left coronary artery ligation was performed in adult male Wistar rats (250 to 300 g; obtained from Harlan-Winkelmann, Borchen, Germany) as described previously. The objective of our study was to analyze whether long-term pharmacological intervention improves endothelial dysfunction and platelet activation at the chronic stage of severe CHF. Structural postinfarction left ventricular remodeling is a dynamic time-dependent process, and we chose the time point of 10 weeks to make sure that the animals were in the chronic stable phase of heart failure. After 10 weeks, hemodynamic studies were performed and infarct size was determined. To exclude potential beneficial effects of rosuvastatin on hemodynamics biasing our results, only animals with elevated left ventricular end-diastolic pressure (LVEDP) and impaired left ventricular function were included during analysis. Pulmonary edema was assessed by net fluid weights. The lungs were then placed in a drying oven for 1 week at 40°C, and the difference between the wet and dry weights yielded the pulmonary fluid accumulation values.

Vascular Reactivity Studies

The descending thoracic aorta was dissected after removal of the heart and cleaned of connective tissue. One section was used for measurement of superoxide production, and the other was cut into 10-μm-thick sections and placed on a glass slide. HE was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. Images were obtained with a Bio-Rad MRC-1024 laser-scanning confocal microscope equipped with a krypton/argon laser. Aortic rings from CHF animals and control tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from CHF and control specimens. Fluorescence was detected with a 585-nm long-pass filter.

Platelet Preparation and Flow Cytometry

Blood was directly taken into a syringe prepared with sodium citrate (3.8%), diluted with PBS (free of Ca2+ and Mg2+) and enriched with d-glucose [5.5 mmol/L] and 0.5% BSA, and incubated with a polyclonal rabbit anti–P-selectin (CD62P) antibody (Becton Dickinson) for 10 minutes at room temperature, followed by incubation with a fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody (Jackson ImmunoResearch) for determination of surface-expressed P-selectin. Staining of the samples was also performed only with the FITC-conjugated secondary antibody in the absence of the primary antibody. Platelet glycoprotein (GP) 53 (CD63) was determined using a mouse anti-rat CD63 monoclonal antibody and consecutively incubated with an FITC-conjugated anti-mouse IgG antibody (both from Becton Dickinson). Platelets within the leukocyte population were identified by the platelet-specific antigen CD42, the expression of which was not modulated in the investigated animals, using an anti-rat CD42 monoclonal FITC-conjugated anti-body (Becton Dickinson) to assess platelet leukocyte adhesion. 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.

Study Substances

Unless stated otherwise, all chemicals were obtained from Sigma in the highest purity available.

Measurement of Superoxide Anion Formation

Vascular superoxide formation was measured using lucigenin-enhanced chemiluminescence. The light reaction between superoxide and lucigenin (5 μmol/L) was detected in a luminometer (Wallac) during incubation of rings in a HEPES-modified Krebs buffer, pH 7.40. The specific chemiluminescence signal was expressed as counts per minute per milligram dry weight of tissue (cpm/mg).

The oxidative fluorescent dye hydroethidine (HE) was used to evaluate in situ production of superoxide as described previously. Unfixed frozen ring segments were cut into 10-μm-thick sections and placed on a glass slide. HE was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. Images were obtained with a Bio-Rad MRC-1024 laser-scanning confocal microscope equipped with a krypton/argon laser. Aortic rings from CHF animals and control tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from CHF and control specimens. Fluorescence was detected with a 585-nm long-pass filter.
by Tukey post hoc test where appropriate; \( P < 0.05 \) was considered statistically significant.

**Results**

**Hemodynamics and Global Data**

Global parameters and hemodynamic measurements of CHF rats and Sham-operated animals are shown in Table 1. Infarct sizes were comparable among the different experimental groups. Because only rats with CHF and increased LVEDP after coronary ligation display endothelial dysfunction and enhanced platelet activation,8 improvement of hemodynamic function by rosuvastatin may potentially influence the results. Therefore, for determination of rosuvastatin effects on vasculature and platelets, only animals with overt CHF, as defined by reduced blood pressure, increased LVEDP, and reduced left ventricular contractility despite treatment, were analyzed.

**Superoxide Formation**

Aortic superoxide production was significantly increased in rats with CHF and normalized by rosuvastatin (Figure 1A). Representative ethidium bromide–stained images of superoxide formation in vascular rings demonstrated increased signal intensity in CHF versus Sham animals, which was markedly reduced in rats treated with rosuvastatin (Figure 1B).

**Vascular Reactivity**

Phenylephrine-induced vasoconstriction was significantly increased in rats with CHF and partly normalized by treatment with rosuvastatin (Figure 2A). Administration of acetylcholine in cumulative doses (Figure 2B; Table 2) induced an endothelium-dependent vasorelaxation, which was impaired in CHF and significantly improved by rosuvastatin treatment. Endothelium-independent vasorelaxation induced by DEA-NONOate did not significantly differ between the groups (Figure 2C; Table 2).

Inhibition of tonic NO release by l-NNA in slightly preconstricted aortic rings induced an additional contraction, which was attenuated in animals with CHF, indicating a reduction of NO bioavailability in CHF aortae. In rosuvastatin-treated CHF animals, l-NNA induced constriction was increased to levels comparable with Sham-operated rats (Figure 2D).

**Platelet VASP Phosphorylation**

Because endothelial function was significantly impaired, we assessed NO-mediated signaling toward platelets. We used in vivo phosphorylated platelet VASP in whole blood, which was immediately fixed after collection, to determine intraluminal NO bioavailability in the living animal. Basal platelet VASP phosphorylation at Ser239 was significantly reduced in CHF and normalized in rats treated with rosuvastatin (Figure 3). Phosphorylation of VASP26 is not only an established marker for NO bioavailability, but also an essential regulatory component in inhibition of platelet activation.4

**Platelet Activation**

Platelet degranulation determined by expression of P-selectin (CD62P; Figure 4A) and glycoprotein 53 (CD63; Figure 4B)

### Table 1. Global Parameters in CHF Rats 10 Weeks After MI Compared With Sham-Operated Animals

<table>
<thead>
<tr>
<th></th>
<th>Sham Placebo</th>
<th>CHF Placebo</th>
<th>CHF Rosuvastatin</th>
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<tr>
<td></td>
<td>( n=9 )</td>
<td>( n=22 )</td>
<td>( n=15 )</td>
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<tr>
<td>SAP, mm Hg</td>
<td>134±5</td>
<td>105±3*</td>
<td>104±3*</td>
</tr>
<tr>
<td>DAP, mm Hg</td>
<td>103±6</td>
<td>78±4*</td>
<td>83±3*</td>
</tr>
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<td>LVEDP, mm Hg</td>
<td>6±1</td>
<td>24±3*</td>
<td>23±2*</td>
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<tr>
<td>( \text{dP/dt}_{\text{max}} \times 1000 \text{mm Hg/s} )</td>
<td>15.2±1.3</td>
<td>8.9±0.5*</td>
<td>9.2±0.5*</td>
</tr>
<tr>
<td>( \text{dP/dt}_{\text{max}} \times 1000 \text{mm Hg/s} )</td>
<td>11.2±1.3</td>
<td>6.3±0.5*</td>
<td>6.9±0.5*</td>
</tr>
<tr>
<td>Lung fluid weight, mg/kg body weight</td>
<td>3.3±0.2</td>
<td>7.0±0.8*</td>
<td>8.0±0.9*</td>
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<tr>
<td>Right ventricle, mg</td>
<td>170±10</td>
<td>360±20*</td>
<td>330±30*</td>
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<tr>
<td>Body weight, g</td>
<td>360.3±18.0</td>
<td>357.0±12.4</td>
<td>343.5±8.7</td>
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Rats were treated with either placebo or rosuvastatin.
SAP indicates systolic arterial pressure; DAP, diastolic arterial pressure.
* \( P < 0.05 \) vs Sham Placebo.

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**Figure 1.** A, Superoxide production in aortic rings detected by lucigenin-enhanced chemiluminescence in Sham- or CHF-rats treated with placebo or rosuvastatin (Rosu). \( ^* P < 0.05 \) vs Sham; \( ^* P < 0.05 \) vs CHF Placebo; \( n=10 \) to 16. B, Representative images of vessels labeled with the superoxide sensitive fluorescent dye HE, which produces a fluorescence when oxidized to ethidium bromide by superoxide.
on the platelet surface was significantly enhanced in CHF, which was reversed by HMG-CoA reductase inhibition. Platelet surface expression of P-selectin plays a pivotal role in platelet–leukocyte interaction. Therefore, the amount of circulating platelet–leukocyte aggregates was determined in whole blood. Platelet–leukocyte aggregation was increased in CHF and reduced by rosuvastatin treatment (Figure 4C). Typical flow cytometry histograms show the rightward shift in P-selectin surface expression in platelets from CHF rats, which was reversed by rosuvastatin treatment (Figure 4D).

Discussion

In this study, we demonstrated that HMG-CoA reductase inhibition reduces platelet activation in experimental CHF. Improved endothelial function and platelet VASP phosphorylation suggest that enhanced endovascular NO bioavailability contributes to statin effects on platelets in CHF.

Patients with CHF experience increased stroke risk.27–29 Therefore, thromboembolic events play a substantial role in determining the morbidity and mortality of CHF populations. The risk of stroke increases with decreasing left ventricular ejection fraction.9 Soluble and platelet-bound adhesion molecules such as P-selectin or CD40-ligand were found to be increased in patients with CHF.7,11,30 Enhanced binding of fibrinogen on glycoprotein IIb/IIIa and surface expression of adhesion molecules further promote the interaction of activated platelets with other platelets, leukocytes, and the endothelium. Increased numbers of circulating platelet aggregates have been well described in patients with heart failure.10 We recently described enhanced platelet activation in an experimental model of CHF after coronary ligation in otherwise healthy rats.8 In our model, subsequent systemic changes depend on the extent of left ventricular dysfunction and are unaffected by possible pre-existing thrombi or vascular dysfunction as in models of spontaneously developing CHF. Because analyses are performed in a chronic disease state, the acute operative procedure per se does not influence the measurements, which were performed 10 weeks after coronary ligation. Platelet surface expression of P-selectin

<table>
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<tr>
<th>TABLE 2. Vasomotor Function in CHF Rats 10 Weeks After MI Compared With Sham-Operated Animals</th>
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<tbody>
<tr>
<td>Agonist</td>
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<tr>
<td>--------</td>
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<tr>
<td>Acetylcholine</td>
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<td></td>
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<td></td>
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<tr>
<td>DEA-NONOate</td>
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Rats were treated with either placebo or rosuvastatin. R₅₀ indicates maximum relaxation. *P<0.01 vs Sham placebo; †P<0.01 vs CHF placebo.
was similar in Sham-operated rats compared with age-matched controls, and no platelet activation was observed in MI animals not developing left ventricular failure.8 In the present study, we showed that platelet degranulation and platelet–leukocyte aggregation were increased in our model of CHF in normocholesterolaemic rats and that this platelet activation was significantly reduced by rosuvastatin treatment irrespective of improvement in LV function.

Multiple effects of statin treatment have been described in patients with, and experimental models of, hypercholesterolemia, including reversal of hypercholesterolemia-associated platelet activation, reduction of platelet reactivity, thrombocyte biosynthesis, thrombin generation, aggregation, and thrombogenic potential,31,32 as well as platelet thrombus formation.33 In addition to positive modulation after initiation of statin treatment, platelet hyperreactivity after its discontinuation has also been described.34 Pleiotropic effects of statins that could positively influence platelet activation include the lowering of tissue factor and thrombin generation at cellular levels.35 The combination of pravastatin with an angiotensin-converting enzyme inhibitor significantly decreased the platelet response to thrombin in atherosclerosis-prone monkeys.36 Cholesterol-independent effects of statins have been reported for experimental and clinical stroke prevention.37

In CHF, impaired left ventricular function subsequently results in less shear stress on the luminal side of the vascular endothelium, which is one of the physiologically most important regulators of eNOS expression and activation in vivo.38 In the CHF model of chronic MI, endothelial dysfunction is associated with reduced systemic NO bioavailability and phosphorylation of platelet VASP.8 VASP phosphorylation reflects the bioactivity/integrity of platelet inhibitors/-inhibitory pathways, including the predominant NO/cGMP pathway.26,39–42 Phosphorylation of VASP is an essential regulatory component in inhibition of platelet activation. Phosphorylation of VASP correlates closely with inhibition of fibrinogen binding to the GP IIb/IIIa receptor.39,40,42,43 NO induces phosphorylation of VASP by NO-dependent activation of guanylyl cyclase and subsequent cGMP-mediated stimulation of cGMP-dependent kinases.26 The relevance of this signaling pathway is underlined by experimental studies demonstrating increased platelet adhesion and aggregation as well as increased levels of P-selectin expression in VASP-deficient mice.39,44 Inhibition of NO formation results in reduced platelet VASP phosphorylation accompanied by increased platelet activation, which can be reversed by exogenous NO.4 In addition, platelets are less sensitive to NO in CHF.45

The pleiotropic effects of HMG-CoA reductase inhibitors in general include improvement of endothelial function, platelet function, atherosclerotic plaque stability, and suppression of vascular inflammation.18,32 Statins exert several protective effects on the endothelium, including reduced activity of NAD(P)H oxidases,46 enhanced expression of vascular eNOS,47 and increased endothelial NO bioavailability.48 Acute administration of statins significantly increased flow-mediated dilation of the brachial artery in healthy normocholesterolaemic subjects,49 and rapid effects of statins on coronary endothelial function have been reported.50,51 Statins prevented the angiotensin II–induced production of ROS in the rat aorta52 and enhanced endothelial function in healthy animals, including cholesterol-independent effects on NO bioavailability.46 In our study, statin-mediated improvement of LV function is not responsible for the observed effects on endothelial function because only animals with CHF despite treatment were included in the study. The normalization of platelet VASP phosphorylation suggests that the reduction of platelet reactivity by rosuvastatin is related to the improvement of endoluminal NO bioavailability.53,54 Improved survival in CHF patients taking statins has been reported;13,14 however, prospective studies are still lacking. Because stroke is a major complication in CHF, contributing to increased morbidity and mortality, reduced platelet activation by statins in CHF, as observed in the present study, adds a new potential mechanism by which statins may improve the prognosis of CHF patients.

In this study of experimental CHF, rosuvastatin significantly improved NO bioavailability and reduced platelet...
activation independent from changes on left ventricular function.

Acknowledgments

This study was partly supported by a grant from AstraZeneca and by the Deutsche Forschungsgemeinschaft DFG (SFB355, B10, C3). The authors wish to thank Heidi Scheuermann, Meike Leutke, and Konrad Rammelt for expert technical assistance.

References


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*Arterioscler Thromb Vasc Biol.* 2005;25:1071-1077; originally published online March 10, 2005;
doi: 10.1161/01.ATV.0000161926.43967.df

*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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