Glucose-Regulated Protein 78 and Platelet Deposition

Effect of Rosuvastatin

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Objective—To investigate the effect of rosuvastatin on platelet deposition under controlled shear rate conditions and to identify new platelet proteins involved in the interaction with the activating substrate.

Methods and Results—Platelet-vessel wall interaction and thrombosis take place under dynamic conditions involving the interaction of the exposed damaged vascular wall with the circulating blood cells and proteins. Blood was perfused over type I collagen at different wall shear rates, and platelet deposition was measured by confocal microscopy. Perfused effluent blood was collected, platelets were sequentially extracted based on differential protein solubility, and proteins were separated by 2D gel electrophoresis. Blockade of 3-hydroxy-3-methylglutaryl–coenzyme A reductase significantly reduced platelet deposition and modulated the expression pattern of 18 proteins in the platelet subproteome. Among them, an increase in platelet surface 78-kDa glucose-regulated protein (GRP78), a stress-inducible multifunctional endoplasmic reticulum protein, was clearly apparent. Immunoprecipitation of platelet GRP78 revealed its interaction with tissue factor. Moreover, blockade of surface GRP78 resulted in a substantial increase in platelet deposition and tissue factor procoagulant activity and in a decrease in clotting time.

Conclusion—These findings demonstrate that blockade of 3-hydroxy-3-methylglutaryl–coenzyme A reductase reduces platelet deposition and inhibits GRP78 translocation from the platelet surface after shear and collagen activation. For the first time to our knowledge, this study reports on the presence and functional role of GRP78 in platelets and indicates that GRP78 has additional functions beyond those of a molecular chaperone. (Arterioscler Thromb Vasc Biol. 2010; 30:1246-1252.)

Key Words: platelets ▪ thrombosis ▪ glucose-regulated protein 78 ▪ statins ▪ thrombosis ▪ tissue factor

The first response to vascular injury consists of platelet adhesion to the damaged vessel wall or to exposed tissue components and is mediated by flow-regulated interactions that have a key influence on subsequent thrombus growth, often culminating in life-threatening complications.1

Clinical results strongly suggest that the beneficial effects of statins (3-hydroxy-3-methylglutaryl–coenzyme A [HMG-CoA] reductase inhibitors) may also be related to factors beyond lowering systemic lipid levels.2,3 The effects of statins are mediated via blockade of mevalonate formation, which results in reduction of cholesterol synthesis. The inhibition of mevalonate formation also results in reduced levels of isoprenoid derivatives, which play important roles in the isoprenylation of small GTPases of the Rho/Rac/Cdc42 family.3–6 Isoprenylation facilitates activation and translocation of these proteins from the cytosol to the plasma membrane and thereby supports the function of these intracellular signaling pathways, which are necessary for many cellular processes in the cardiovascular system.4,7,8 Indeed, statins inhibit cell proliferation, improve endothelial function, reduce inflammation, stabilize the atherosclerotic plaque, and enhance fibrinolysis.5,9 Although contradictory findings about the effect of different statins on platelet inhibition have been reported, there is evidence for their antithrombotic properties,9,10 even in the venous side.11

Therefore, our purpose in the present study was to evaluate the short-term effect of rosuvastatin, a newly available HMG-CoA reductase inhibitor that seems to have higher lipid-lowering efficacy than other statins on platelet function under flow conditions. A proteomic analysis was performed to identify new potential platelet proteins modulated by the inhibition of HMG-CoA reductase involved in platelet-vessel wall interactions. For the first time to our knowledge, we report on the presence of a 78-kDa glucose-regulated protein (GRP78) in the platelet surface and its changes by shear stress. Our results also indicate that rosuvastatin induces inhibition of flow-dependent platelet deposition and inhibits GRP78 translocation from the platelet surface, even after platelet activation.
GRP78 may play a role in thrombosis, beyond its function as an endoplasmic reticulum (ER) chaperone.

Methods

Blood was perfused over type I collagen at different wall shear rates, and platelet deposition was measured by confocal microscopy.13 Perfused effluent blood was collected, platelets were sequentially extracted based on differential protein solubility, and proteins were separated by 2D gel electrophoresis. Western blot and immunofluorescence analysis were performed to study GRP78 expression in platelets. In addition, flow cytometry, tissue factor (TF) procoagulant activity, immunoprecipitation, and thromboelastographic studies were performed. Data are given as mean±SEM unless otherwise indicated. An expanded methods section is available in the Supplemental Material (available online at http://atvb.ahajournals.org).

Results

Effect of Rosuvastatin on Shear-Induced Platelet Deposition and Glycoprotein IIb–IIIa Activation

In vitro rosuvastatin treatment significantly reduced platelet deposition triggered by collagen in porcine blood at both low and high wall shear rates. At 250 s⁻¹, platelet deposition was significantly reduced by rosuvastatin (P<0.001) (Figure 1A). The surface covered by platelets was reduced from 5134±350 μm² per field (nontreated blood) to 2557±225 μm² per field (rosuvastatin, 2 μmol/L), 2702±260 μm² per field (rosuvastatin, 4 μmol/L), and 2062±206 μm² per field (rosuvastatin, 8 μmol/L). Platelet deposition decreased by 50%, 47%, and 60%, respectively.

Rosuvastatin also significantly reduced thrombus height (Figure 1B) at the low wall shear rate. The highest thrombi observed in the proximal zone of the flow path coated with collagen decreased from 21.02±0.77 μm (nontreated blood) to 14.35±0.90 μm (rosuvastatin, 2 μmol/L), 16.46±1.00 μm (rosuvastatin, 4 μmol/L), and 16.13±1.00 μm (rosuvastatin, 8 μmol/L). Thrombus height was significantly reduced by 30% (P<0.01).

At 1500 s⁻¹, the surface covered by platelets significantly decreased from 8561±1283 μm² per field in nontreated blood to 3763±880 μm² per field in blood treated with rosuvastatin, 8 μmol/L, as shown in Figure 1C, which means a 56% reduction on platelet deposition (P<0.005). Furthermore, rosuvastatin was able to significantly reduce platelet adhesion in conditions in which coagulation was fully prevented and in conditions in which coagulation was triggered, with similar reduction levels in both conditions (Supplemental Figure I).

Flow cytometry analysis showed that rosuvastatin, 8 μmol/L, significantly reduced the extent of activation of glycoprotein (GP) IIb–IIIa on ADP-induced platelet activation (P<0.05), as shown in Figure 1D.

Proteome Changes in Rosuvastatin-Treated Platelets

Differential proteomic analysis was used to identify changes induced by rosuvastatin in effluent sheared porcine platelets from flow experiments run at 1500 s⁻¹. Average gels were obtained from pooling at least 3 independent experiments. After a first screening using a broad pH range (from 3 to 10), most changes induced by rosuvastatin were localized in a pH ranging from 5 to 8, which led us to study the proteome in a pH ranging from 5 to 8. Proteomic analysis revealed that rosuvastatin, 8 μmol/L, modulated the expression pattern of 10 spots in the cytosolic fraction and 8 spots in the membrane-enriched fraction (Supplemental Table I and Supplemental Figure II).

Identification of GRP78 and Changes Induced by Rosuvastatin

Among the different proteins modulated by rosuvastatin treatment, we identified GRP78 as a series of 3 spots (region 3 of Supplemental Figure II) with a relative molecular mass of approximately 72 kDa, clustered around pH 4.8 and 5.2 and identified by matrix-assisted laser desorption ionization-time of flight analysis as GRP78/BiP protein (accession No. AAF13605; Supplemental Figure III). GRP78 seemed to increase in the urea-soluble fraction by decreasing in the Tris-soluble fraction when platelets were treated with rosuvastatin. Rosuvastatin treatment apparently induced an increase of GRP78 expression in the urea-soluble fraction.
(approximately 30%) and a reduction of the expression in the Tris-soluble fraction (approximately 25%) in the 3 spots identified as GRP78 (Figure 2A and B).

To confirm the changes observed by 2D analysis, we performed Western blot analysis from 1D gels of protein extracts from platelets subjected to the different treatments. Western blot analysis demonstrated an increase of GRP78 in the urea-detergent–soluble fraction in rosuvastatin-sheared platelets compared with control sheared platelets ($P < 0.05$), as shown in Figure 2C. To further elucidate the effect of rosuvastatin on GRP78, both resting and postperfusion sheared nonpermeabilized human platelets were analyzed by confocal microscopy with antibodies against GPIIa (CD61) and GRP78. In resting platelets, significant amounts of GRP78 were detected in the platelet surface. In sheared platelets (effluent postperfusion platelets), membrane GRP78 was weaker than in resting platelets. In contrast, sheared platelets that had been preincubated with rosuvastatin showed levels of surface GRP78 similar to resting platelets (Figure 3).

**Effect of GRP78 Blockade on Platelet Deposition and Coagulation**

To investigate the functional role of GRP78 in platelet adhesion, a further set of 3-minute perfusion experiments at $1500 \text{s}^{-1}$ with human heparinized blood was performed in the presence of $10 \mu\text{g/mL}$ of anti-GRP78 or nonimmune goat IgG as control before rosuvastatin treatment. Blockade of GRP78 resulted in a substantial increase in platelet deposition ($P < 0.001$) compared with control samples, as shown in Figure 4A. Blockade of GRP78 in rosuvastatin-treated platelets also resulted in a significant increase in platelet deposition.

Interestingly, GRP78 blockade also resulted in a shortened clotting time ($91 \pm 6$ seconds versus $81 \pm 6$ seconds, control versus blockade of GRP78; $P < 0.05$), analyzed by thromboelastometry with the EXTEM assay, which measures the contribution of the extrinsic pathway of coagulation to clot formation (Figure 4B). Accordingly, blockade of GRP78 significantly increased tissue factor (TF)-procoagulant activity (PCA) ($P < 0.05$) in whole blood membrane extracts. Furthermore, as shown in Figure 4C, blockade of GRP78 significantly increased TF-PCA in platelet-rich plasma (PRP) but not in platelet-poor plasma (PPP).

**GRP78/TF Interaction**

TF interacted with GRP78, as observed by GRP78 immunoprecipitation. Both total platelet lysates and platelet membrane fractions of resting untreated platelets showed interaction with TF (Figure 5A). We did not detect the presence of GPVI, GPIb, GPIV, or GPIIIa on GRP78 immunoprecipitates.

GRP78-TF colocalization in human platelets was analyzed by immunofluorescence. We observed that GRP78 colocalized with TF in platelets that adhered to collagen and in effluent sheared platelets. The increase in platelet surface GRP78 induced by rosuvastatin colocalized with TF. Indeed, a double immunostaining of TF and GRP78 in effluent sheared platelets showed that in control platelets $13.2 \pm 3.0\%$ of the area positively stained for TF colocalized with the area that stained positively for GRP78; in rosuvastatin-treated samples, $54.7 \pm 12.0\%$ of the area stained positively for TF colocalized with GRP78 (Figure 5B) ($P < 0.05$). Similarly, when analyzing platelets that adhered to collagen, in control samples, $23.9 \pm 3.7\%$ of platelets positively stained for TF colocalized with GRP78; in rosuvastatin-treated samples (with total platelet deposition significantly reduced), this percentage increased up to $45.1 \pm 5.9\%$ ($P < 0.01$) (Figure 6). Therefore, rosuvastatin maintained high GRP78 expression in the platelet surface that colocalized with TF.

**Figure 2.** Effect of rosuvastatin on GRP78 expression in platelets by 2D and 1D. A, Enlarged images of region 3 of 2D gels containing spots identified as GRP78. B, Increase/decrease of the 3 spots identified as GRP78 in sheared platelets treated with rosuvastatin. Data are given as percentage of change of GRP78 spots from sheared platelets treated with rosuvastatin vs sheared untreated platelets ($n=3$). Statistical analysis was performed by Mann-Whitney test ($P < 0.05$). C, Western blot analysis of GRP78 in the urea-detergent–soluble fraction of sheared and unsheared platelets. Data are given as mean±SEM arbitrary units normalized by $\beta$-actin ($n=3$). Statistical analysis was performed by ANOVA ($P < 0.05$).
In the present study, we demonstrate that HMG-CoA reductase inhibition reduces platelet-collagen interaction by reducing platelet adhesion and thrombus height under flow conditions and maintains GRP78 on the platelet surface. The postperfusion confocal analysis allowed the study of thrombus formation on a thrombogenic substrate, such as collagen, by measuring platelet deposition and thrombus height. Rosuvastatin was able to reduce platelet deposition at both venous and arterial wall shear rates. Because HMG-CoA reductase inhibitors have been shown to exert several cellular effects through reduction of isoprenoid derivatives, we aimed to identify novel target proteins modified by statin treatment on platelets. Blockade of HMG-CoA reductase modulated the expression pattern of 10 proteins in the cytosolic fraction and 8 proteins in the membrane-enriched fraction. Most of the identified proteins differentially modulated by rosuvastatin,

**Figure 3.** Effect of rosuvastatin on GRP78 distribution on non-permeabilized human platelets. Representative confocal images of platelet surface GRP78 (red) and platelet membrane receptor GPIIa (green). I indicates control unsheared platelets; II, control sheared platelets (1500 s⁻¹); and III, rosuvastatin, 8 µmol/L, sheared platelets. The bar indicates 1 µm.

**Figure 4.** Effect of blocking GRP78 on platelet deposition, clotting time, and TF-PCA. A, Mean platelet deposition (MPD). Statistical analysis was performed by ANOVA (*P<0.001 vs control and rosuvastatin; **P<0.05 vs control, GRP78 blockade, and GRP78 plus rosuvastatin; and #P<0.05 vs all) (n=10). B, Clotting time. Statistical analysis was performed by paired t-test analysis (*P<0.05 vs control) (n=5). C, TF-PCA of whole blood extracts, PRP, and PPP samples. Data are given as percentage of change vs control samples. Statistical analysis was performed by paired t-test analysis (*P<0.05 vs control) (n=5).

**Discussion**

In the present study, we demonstrate that HMG-CoA reductase inhibition reduces platelet-collagen interaction by reducing platelet adhesion and thrombus height under flow conditions and maintains GRP78 on the platelet surface.

The postperfusion confocal analysis allowed the study of thrombus formation on a thrombogenic substrate, such as collagen, by measuring platelet deposition and thrombus height. Rosuvastatin was able to reduce platelet deposition at both venous and arterial wall shear rates. Because HMG-CoA reductase inhibitors have been shown to exert several cellular effects through reduction of isoprenoid derivatives, we aimed to identify novel target proteins modified by statin treatment on platelets. Blockade of HMG-CoA reductase modulated the expression pattern of 10 proteins in the cytosolic fraction and 8 proteins in the membrane-enriched fraction. Most of the identified proteins differentially modulated by rosuvastatin,
such as heat shock protein 70 and vinculin, are already known to be involved in platelet adhesion and spreading.14,15 However, we report herein for the first time, to our knowledge, that platelets have GRP78 in both the membrane and the cytosol. GRP78 is translocated from the platelet membrane by shear forces, and rosuvastatin inhibits this translocation in shear-activated platelets.

GRP78 is a multifunctional ER protein that participates in many cellular processes, such as translocation of newly synthesized polypeptides across the ER membrane, facilitating the folding and assembly of proteins by preventing aggregation and regulating calcium homeostasis.16–19 Although GRP78 resides in the ER lumen, a number of studies demonstrate that GRP78 is also located on the cell surface.20–22 Indeed, 2 independent groups have detected that a fraction of GRP78 can exist as a transmembrane protein23,24 in Chinese hamster ovary, human leukemia, bladder carcinoma, and human embryonic kidney 293 T cells. These observations are in agreement with our findings. Indeed, we identified GRP78 both in cytosolic- and membrane/cytoskeleton-enriched fractions, and confocal analysis revealed that GRP78 translocated in and out of the membrane, depending on the activation state. On the other hand, there is evidence that GRP78 can be released from intact cells and that it has also been detected in serum samples from healthy subjects.25 In fact, we also identified GRP78 in plasma from effluent blood (data not shown), which means that platelet GRP78 could eventually be released.

GRP78 is preferentially expressed in advanced atherosclerotic lesions26 and on the fibrous cap surface in apolipoprotein E–deficient mice.27 A recent study28 has shown that atheroprone, but not atheroprotective flow upregulated GRP78 in endothelial cells via a p38 mitogen-activated protein kinase and α2β1-dependent mechanism, and that increased GRP78 correlated with activation of the unfolded protein response, providing a protective compensatory effect in response to ER stress. We observed by confocal microscopy analysis that GRP78 localization in platelets appeared to depend on stimulation by shear rate and collagen. Membrane GRP78 expression was higher in resting platelets than in effluent platelets, suggesting that, on the shear-induced activation surface, GRP78 could have been either partially released or translocated inside the platelet. Interestingly, rosuvastatin-treated platelets maintained surface GRP78 expression after exposure to shear, probably indicating inhibi-
tion of shear-induced translocation of GRP78. This effect was also observed by 2D and Western blot because GRP78 expression was significantly increased in the urea-enriched fraction. The urea-enriched fraction not only contains the external membrane fraction but also the cytoskeleton fraction, which may account for the slightly different GRP78 patterns observed by 1D and 2D and confocal microscopy in sheared platelets.

It has recently been shown that statins activate the unfolded protein response and induce protective GRP78 expression in macrophages. In line with these findings, we observed that in normcholesterolemic animals, treatment with rosuvastatin also increased platelet GRP78 (both cytoplasm and membrane fractions; data not shown), thus suggesting a possible effect on megakaryocytes that is transmitted to the platelets. Further research in this area seems warranted.

GRP78 provides dual functions in either the ER or the cell membrane, where it has multiple binding partners. Herein, we show that blockade of GRP78 in flow experiments markedly increased platelet deposition and TF-PCA and significantly reduced clotting time, supporting a protective role of surface GRP78. Rosuvastatin was able to partially inhibit the increase in platelet deposition induced by GRP78 blockade, indicating that it exerts additional protective functions in addition to translocating GRP78 to the membrane. In fact, we observed an additional inhibitory effect of rosuvastatin beyond GRP78 effects because we found that rosuvastatin decreased GPIIb-IIIa activation in ADP-induced activated platelets.

Cell surface–associated GRP78 has been speculated to serve a protective role by inhibiting TF through direct binding to the endothelium overlying the plaque. Indeed, we observed a shortened clotting time and increased procoagulant activity in whole blood membrane extracts and in PRP samples when blocking GRP78. Intriguingly, GRP78 blockade did not increase TF-PCA in PPP samples. Moreover, we found that platelet GRP78 actively interacted with TF. Confocal analysis of adhered and effluent platelets showed a greater degree of colocalization of GRP78 and TF in rosuvastatin-treated platelets. Because we observed that rosuvastatin maintained high levels of GRP78 on the platelet surface, the inhibitory effect of rosuvastatin in platelet adhesion could be attributed to reduced TF activity as the result of direct binding of surface GRP78 to TF. The role of surface GRP78 on thrombus growth could be related to its ability to attenuate TF activity or might be attributed to additional unknown mechanisms directly involved in platelet activation, which will require future studies.

In summary, our data indicate that rosuvastatin inhibits platelet deposition under flow conditions and maintains platelet surface GRP78 in shear-activated platelets. Our results also show that GRP78 interacts with TF, blocking its procoagulant activity and that GRP78 blockade increases platelet deposition and reduces clotting time, suggesting a protective effect of GRP78 against thrombus formation.

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Disclosures
None.

References


Supplemental material to:

**GRP78 And Platelet Deposition: Effect Of Rosuvastatin**

Running title: *GRP78, platelets, and rosvastatin*

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MATERIALS AND METHODS

Experimental design

Blood was obtained from non-smoking healthy donors, who denied taking any antiplatelet medication for 15 days prior to blood extraction. Procedures were approved by the Clinical Research Committee of our Institution. Blood was withdrawn in 10 UI/ml sodium heparin by cubital venipuncture. Alternatively, when indicated blood was withdrawn into 1:10 (vol/vol) 90 mmol/L trisodium citrate.

In some experiments, white commercial fixed-breed pigs (weight ≈ 46 kg) were used and all procedures were in accordance with NIH guidelines and followed the American Physiological Society Guidelines for animal research. Porcine blood was withdrawn into 1:10 (vol/vol) 90 mmol/L trisodium citrate by femoral venipuncture, kept at 20 °C, and used within 2 hours of collection.

Platelet count, leukocyte count, and hematocrit were all within normal ranges.
Platelets were rendered fluorescent by the addition of mepacrine 10 μmol/L (Sigma), unless otherwise specified. Blood was then incubated at 37 °C for 15 min with rosvastatin (Astra Zeneca). When indicated blood was incubated in the presence of 10 μg/ml of anti-GRP78 (Santa Cruz Biotechnology) for 20 min at 37 °C prior incubation with rosvastatin.

Flow experiments in flat chamber

Type I collagen-coated slides were prepared and placed in a parallel plate chamber described in detail elsewhere. The flow chamber was assembled and filled with Tyrode’s buffer (134 mmol/L NaCl, 0.34 mmol/L Na₂HPO₄x12H₂O, 2.9 mmol/L KCl, 12 mmol/L NaHCO₃, 1 mmol/L MgCl₂x6H₂O, 20 mmol/L Hepes). A peristaltic pump was used to perfuse the buffer through the chamber. After 1 min buffer preperfusion, blood was introduced into the chamber at constant shear rates of 250 s⁻¹ and 1500 s⁻¹ for 5 min, unless otherwise indicated. At the end of blood perfusion, buffer was
again circulated for 1 min through the chamber under identical flow conditions. The entire system was kept at 37 °C. After perfusions, collagen-coated slides were carefully removed from the system, rinsed with PBS pH 7.4 and fixed with 3.8 % paraformaldehyde for 15 min. Fixed slides were then washed with PBS and mounted on glass slides with Glycerol Mounting Medium (Dako Cytomation).

For experiments in the absence of coagulation, blood was collected into 40 μM D-Phe-Pro-Arg-chloromethyl ketone (PPACK). For experiments in which coagulation was afterwards triggered, blood was collected in 10 % 90 mmol/L trisodium citrate. After labeling with mepacrine, blood was perfused for 3 min at 1500 s⁻¹. For coagulation experiments, citrate-anticoagulated blood was perfused with 10 % (v/v) Hepes buffer (5 mmol/L Hepes, 136 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH₂PO₄ and 1 % BSA, pH 7.45) containing 200 mmol/L CaCl₂ and TF (Innovin, 5mg/mL), as described by Munnix et al³.

**Measurement of platelet deposition**

Platelet deposition on the surface of collagen was scanned with a Leica TCS SP2 confocal laser scanning microscope. A 488 nm Ar Kr-laser was used as light source. Platelets were viewed with a HCX PL APO 20X / 0.7 IMM CORR objective. Five fields along the adhesion surface were systematically acquired for total platelet deposition analysis, discarding entrance and exit of the flow path (field: 750 μm x 750 μm). A threshold was applied to distinguish platelets from the background, and the same value was then used for analyzing all the stacks of confocal images collected for a given experiment. Digital color images were converted into black-and-white images. The surface covered by platelets was calculated using NIH Image software (public domain software by Dr Wayne Rasband, National Institutes of Health, version 1.62). Mean platelet deposition was expressed as the area covered by platelets per analyzed field (μm²/field). To measure thrombus height an image stack was recorded from the xz-sections in the y-direction. The projection axis was the orthogonal axis.
(y-axis for vertical xz-sections). Thrombus height was measured from the maximum projection of the stack.

**Flow Cytometry**

For flow cytometry analysis of conformational change of GPIIb-IIIa, rosuvastatin-treated samples were diluted 1:10 in Tyrode Buffer. 25 µl aliquots were activated with ADP (0-10 µmol/L, 5 min, 37 °C). A phycoerytrin (PE) conjugated mAb to CD61 (Pharmingen) was used as an activation-independent marker of platelets and GPIIb-IIIa conformational change was assessed with a fluorescein isothiocyanate (FITC) conjugated PAC-1 mAb (Beckton Dickinson). The reaction mixture was incubated in the dark at room temperature for 20 minutes. To assess the extent of non-specific association of proteins with platelets, blood was added to control tubes with FITC-labeled and PE-labeled non-immune immunoglobulins. Platelet population was identified based on its forward and side scatter and the association with CD61 antibody. A total of 10000 events were analyzed for percentage of positive platelets using Expo32 ADC XL 4 Color software. Fluorescence was measured with a Beckman Coulter Epics XL instrument. Fractions of the specific fluorescence-positive platelets were obtained after subtraction of non-specific fluorescence in the samples labeled with non-immune immunoglobulins. All measurements were fluorescence-compensated on a daily basis for each set of measured samples using calibration beads.

**Proteomic Analysis**

**Protein Extraction:** Static and sheared platelets were immediately isolated by low speed centrifugation (250 g, 10 min), at room temperature. Platelet number was
adjusted to 4x10^8 platelets/ml and centrifuged at 1400 g for 15 min to obtain platelet pellets, which were stored deep-frozen (-80 ºC).

Platelets were sequentially extracted based on differential protein solubility. A tris-soluble (cytosolic) fraction and an urea-detergent soluble (cytoskeleton/membrane) fraction were obtained. Briefly, samples were homogenized in 40 mmol/L Tris base buffer, incubated for 15 min, and centrifuged at 16000 g (20 min, 4ºC). Protein pellets were washed once with Tris-buffer and further extracted with an urea/thiourea-chaps buffer (7 mol/L Urea; 2 mol/L Thiourea; 4 % CHAPS; 40 mmol/L Tris-Base) for 15 min, as described above. Prior protein separation by 2-DE, sample contaminants were removed with a commercial kit (2D-CleanUp Kit, Amersham). Protein concentration in the extracts was measured with 2D-Quant Kit (GE-HealthCare) as indicated by the manufacturer.

Two-Dimensional Gel Electrophoresis (2-DE): Isoelectric focusing (IEF) was performed using the Protean-IEF cell (BioRad). The method used was that according to Molloy et al with modifications. In brief, 150 μg of protein were resuspended in 350 μl of rehydratation buffer (7 mol/L urea; 2 mol/L thiourea, 2 % CHAPS, 100 mmol/L DTT, 0.2 % carrier ampholytes) and loaded onto 17 cm dry IPG strips (p 3-10 linear range, BioRad) during 16 h by active rehydratation at 50 V. Proteins were then focused by progressive voltage increase up to 10000 V within 5 hours, with a final step at 10000 V until 70000 Volts Hour. After IEF, strips were equilibrated with a reducing solution (50 mmol/L Tris-HCl buffer, pH 8.8, containing 6 mol/L urea, 2 % SDS, 30 % glycerol, and 2 % DTT) and an alkylating solution (50 mmol/L Tris-HCl buffer pH 8.8, 6 mol/L urea, 2 % SDS, 30 % glycerol, and 2.5 % iodoacetamide), for 15 min. Equilibrated IPG strips were transferred for the second dimension (SDS-PAGE) onto 10% polyacrilamide gels. Electrophoresis was performed using a Protean system (Ettan Dalt, Amersham) at 17 W/gel. Gels were stained with pink
flamingo for compatibility with the subsequent analysis of proteins by mass spectrometry.

**Differential Image Analysis**: Fluorescently stained gels were scanned (Typhoon), and analyzed for differences in protein patterns between groups with the 7.3 PD-Quest software (BioRad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

**Protein Identification by MALDI-TOF-MS Analysis**: Protein spots of interest were excised from 2-DE gels, washed with 50 mmol/L ammonium bicarbonate, 50 % methanol, dehydratated with 100 % acetonitrile, and dried under vacuum before enzymatic digestion with sequence-grade modified porcine trypsin (Promega). Peptides from in-gel-trypsin digestion (1 µl) were mixed 1:1 with 5 mg/ml α-cyano-4-hydroxy-cinnamic acid and spotted on a stainless steel mass spectrometry slide. Protein identification was performed by peptide-mass fingerprint using an Ettan MALDI-TOF Pro matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer (GE-Healthcare) operated in delayed extraction/reflector mode. MALDI-generated mass spectra were internally calibrated using trypsin autolysis products, Ang III (angiotensin III), and ACTH (adrenocorticotropic hormone) peaks. Peptide masses were searched against the National Center for Biotechnology Information nonredundant mammalian database using ProFound™ and were confirmed using a Mascot search from Matrixscience selecting the SwissProt database. Because human protein database is widely more extensive than pig database, identified human proteins were blasted against pig proteins and validated if they reached an identity higher than 80%. Protein identification was based on the measurement of at least 10 peptides with a minimum of 50 % matched peptides and coverage higher than 20 %. Minimal expectation for valid identification was 0.01 and $P<0.05$. 
Western Blot Analysis

Sample extracts (25 μg protein) were resolved by 10 % SDS-PAGE and electrotransferred to nitrocellulose membranes, as described previously.\(^5\) Detection was performed with a goat polyclonal antibody against GRP78 (Santa Cruz Biotechnology) and a mouse monoclonal antibody against tissue factor (TF) (American Diagnostica). Band densities were determined with the ChemiDoc™ XRS system (Bio-Rad) in chemiluminescence detection mode and analyzed with Quantity-One software (Bio-Rad). Normalization was performed against β-actin.

Immunofluorescence

GRP78 expression was analyzed by performing perfusion experiments at a wall shear rate of 1500 s\(^{-1}\) with blood not labeled with mepacrine. Unsheared and effluent blood was collected and fixed with 3.8 % paraformaldehyde for 30 min at room temperature. Platelet-rich plasma was obtained by centrifugation at 200 g for 17 min. Platelets were then isolated by centrifugation, immobilized on poly-L-lysine-coated coverslides overnight, incubated with blocking buffer (1 % bovine serum albumin in PBS), and afterwards incubated with a goat polyclonal GRP78 (c-20) antibody (Santa Cruz Biotechnology) and with a mouse monoclonal CD61-FITC antibody (Pharmingen). Coverslides were washed and incubated with Alexa Fluor 633 rabbit anti-goat IgG (H+L). Immunostained coverslides were washed and covered with Prolong Gold antifade reagent. Images were recorded by fluorescence confocal microscopy (Leica TCS SP2) with a HCX PL APO 63x/1.2 W Corr/0.17 CS objective. Controls with no primary antibody showed no fluorescence labelling. Fluorescent images were acquired in a spatial data set (acquiring z-sections) and the maximum intensity projection was created. The maximum projection was linearly filtered to smooth edges and suppress noise and adjusted for brightness and contrast.
For colocalization analysis of GRP78 and TF on adhered platelets and sheared effluent platelets a further set of flow experiments with unlabeled platelets was performed. After perfusions, collagen-coated slides were rinsed with PBS pH 7.4 and fixed with 3.8 % paraformaldehyde at room temperature, and incubated in blocking buffer. Effluent sheared platelets were collected and GRP78 was immunostained as described above. TF was detected with a mouse monoclonal TF antibody (American Diagnostica) as primary antibody and an Alexa Fluor 488 donkey anti-mouse IgG (H+L) as secondary antibody. Images were recorded by fluorescence confocal microscopy (HCX PL APO 63x/1.2 W Corr/0.17 CS). GRP78 and TF colocalization was analyzed with Metamorph software (Universal Imaging Corp, West Chester, PA).

**GRP78 immunoprecipitation**

Platelet-rich plasma (PRP) was prepared from untreated blood by centrifugation at 250 g for 10 min. To obtain washed platelets, PRP was centrifuged at 1200 g in the presence of PGE$_1$ (0.1 μg/mL) for 10 min at room temperature. Platelets (3x10$^8$ platelets/mL) were washed with Tyrode-Hepes, treated with PGE$_1$ (0.1 μg/mL) and centrifuged at 1200 g for 10 min at 4 °C.

The pellet was lysed with 1 mL of cold lysis buffer (1 % Triton X-100, 10 mmol/L Tris, pH 7.5, 150 mmol/L KCl, and protease inhibitors), and then centrifuged at 10 000 g for 10 min at 4°C. Alternatively, platelet subfractionation was performed as described. Supernatants of platelet lysates were precleared by incubating for 30 min at 4°C in lysis buffer containing Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology). Samples were incubated with GRP78 antibody overnight at 4°C. Antibody–antigen complexes were precipitated with protein A/G PLUS-Agarose and then washed twice with lysis buffer. Proteins were eluted from the resin by heating at 95°C for 5 min in electrophoresis sample buffer.
Thrombelastographic coagulation analysis

Dynamic whole blood clot formation was performed with the ROTEM coagulation analyzer (Pentapharm, Munich, Germany), which is based on the thrombelastograph system using the extrinsically activated Ex-TEM assay (containing 20 µl CaCl$_2$ 0.2 mol/L, 20 µl TF, 300 µl citrated blood). Clotting time (CT) was determined. The ROTEM device was checked for proper functioning according to the manufacturer’s recommendation using a control serum (ROTROL). Tests were performed using ROTEM cups and pins. All reagents were purchased from Pentapharm GmbH.

TF Procoagulant Activity (TF-PCA)

Blood was drawn in trisodium citrate (1:10) and frozen at –80ºC. Samples were subjected to 3 cycles of freezing and thawing to completely lyse all cellular elements. TBS-EDTA buffer was added to the samples and then centrifuged (450,000 g; 15 min.) to pellet all particulate material. Membrane pellets were resuspended in TBS-EDTA buffer and the washing/centrifugation cycle was repeated twice. Pellets were washed in HBSA buffer (137 mmol/L NaCl, 5.38 mmol/L KCl, 5.55 mmol/L glucose, 10 mmol/L HEPES, pH 7.5) and then resuspended in 100 µL of HBSA buffer for further assay of TF-PCA. Alternatively, TF-PCA was also measured in PRP and platelet poor plasma (PPP) samples. TF-PCA was measured by using a commercially available chromogenic FXa generation assay (Actichrome TF, American Diagnostica) following the manufacturer’s instructions.

Statistical analysis

Results were expressed as mean±SEM. After testing for normal distribution and equality of variances with Levene’s $F$ test, Student’s $t$-test or ANOVA as appropriate were used to determine statistical significance between treatments. A value of $P<0.05$ was considered significant.
SUPPLEMENTAL TABLE I: Summary of spots differentially expressed upon rosvastatin treatment. Statistical analysis was performed by Mann-Whitney test of spots with more than 2-fold change (*P<0.05).

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Fraction</th>
<th>Rosuvastatin-induced change (&gt;2-fold change) *</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>HSP70</td>
<td>Tris-soluble</td>
</tr>
<tr>
<td>2</td>
<td>HSP70</td>
<td>Tris-soluble</td>
</tr>
<tr>
<td>3</td>
<td>GRP78</td>
<td>Tris-soluble, Urea-soluble</td>
</tr>
<tr>
<td>4</td>
<td>β actin</td>
<td>Tris-soluble</td>
</tr>
<tr>
<td>5</td>
<td>albumin</td>
<td>Tris-soluble</td>
</tr>
<tr>
<td>6</td>
<td>albumin</td>
<td>Tris-soluble, Urea-soluble</td>
</tr>
<tr>
<td>7</td>
<td>NMHC-9</td>
<td>Urea-soluble</td>
</tr>
<tr>
<td>8</td>
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<td>Tris-soluble</td>
</tr>
<tr>
<td>9</td>
<td>vinculin</td>
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</tr>
<tr>
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</tr>
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<tr>
<td>16</td>
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<td>Urea-soluble</td>
</tr>
</tbody>
</table>
Figure I. Effect of rosuvastatin on platelet adhesion under anticoagulant and coagulation-triggered conditions. Blood was perfused for 3 min at 1500 s⁻¹. Results are expressed as mean values of surface covered by platelets per analyzed field (μm²/field)±SEM (n=5). Statistical analysis was performed by unpaired Student’s t-test. *P<0.05 vs. control.
Figure II. Rosuvastatin-induced modifications in the proteomic profile of sheared porcine platelets. (A): Protein spots in 2-DE gels of the tris-soluble subproteome (cytosol-enriched fraction). (B): Protein spots in 2-DE gels of the urea/detergent-soluble subproteome (membrane and cytoskeleton-enriched fraction).
Figure III. Mass spect of identified GRP78 (A) with matched peptides (B)
REFERENCES


