Phosphorylation of Vasodilator-Stimulated Phosphoprotein Prevents Platelet-Neutrophil Complex Formation and Dampens Myocardial Ischemia-Reperfusion Injury

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Background—Recent work has suggested that the formation of platelet-neutrophil complexes (PNCs) aggravates the severity of inflammatory tissue injury. Given the importance of vasodilator-stimulated phosphoprotein (VASP) for platelet function, we pursued the role of VASP on the formation of PNCs and its impact on the extent of myocardial ischemia-reperfusion (IR) injury.

Methods and Results—In initial in vitro studies we found that neutrophils facilitated the movement of platelets across endothelial monolayers. Phosphorylation of VASP reduced the formation of PNCs and transendothelial movement of PNCs. During myocardial IR injury, VASP<sup>−/−</sup> animals demonstrated reduced intravascular formation of PNCs and reduced presence of PNCs within the ischemic myocardial tissue. This was associated with reduced IR injury. Studies using platelet transfer and bone marrow chimeric animals showed that hematopoietic VASP expression was crucial for the intravascular formation of PNCs and the presence of PNCs within ischemic myocardial tissue and the extent of myocardial IR injury. Furthermore, phosphorylation of VASP on Ser<sup>153</sup> or Ser<sup>235</sup> reduced intravascular PNC formation and presence of PNCs within ischemic myocardial tissue. This finding was associated with reduced myocardial IR injury.

Conclusion—Previously unappreciated, the phosphorylation of VASP performs a key function for the formation of PNCs that is crucially important for the extent of myocardial IR injury. (Circulation. 2011;123:2579-2590.)

Key Words: vasodilator-stimulated phosphoprotein  □ phosphorylation □ ischemia-reperfusion injury □ myocardial infarction

A pproximately 3.8 million men and 3.4 million women die of myocardial ischemia-reperfusion (IR) injury each year. In order to minimize the consequences of coronary vessel occlusion, early reperfusion of the infarcted myocardium is the most effective therapy. This approach reduces the size of a myocardial infarction and improves the clinical outcome. Nevertheless, reperfusion of the ischemic myocardium can induce injury. This phenomenon, termed myocardial ischemia-reperfusion injury, paradoxically reduces the beneficial effects of reperfusion. The reperfusion phase is marked by cellular swelling, contracture of myofibrils, and a disruption of the sarcolemma. This structural derangement is caused in part by neutrophils, which are attracted into the ischemic tissue. Platelets also participate in the process of neutrophil recruitment and aggravate myocardial tissue damage during the reperfusion phase. Finally, these events result in an inflammatory response, which has significant impact on apoptotic events within the myocardium.

Clinical Perspective on p 2590

Recent work has demonstrated that the formation of platelet-neutrophil complexes (PNCs) significantly affects the extent of inflammatory tissue damage. In a study by Zarbock et al, the extent of acute lung injury was significantly reduced by blocking the formation of PNCs. Furthermore, Weismueller et al demonstrated that neutrophils facilitate transepithelial movement of platelets into the intestinal lumen. Firm adhesion between neutrophils and platelets is achieved by fibrinogen binding to CD11b/CD18 on neutrophils and the glycoprotein (GP) IIb/IIIa receptor on plate-
lets. Dynamic changes of the cytoskeleton alter the expression of GP IIb/IIIa on platelets and of CD11b/CD18 on neutrophils. Bennett et al demonstrated that the cytoskeleton determines the affinity of the GP IIb/IIIa receptor to fibrinogen. A key regulatory protein for rapid dynamic changes of the cytoskeleton is vasodilator-stimulated phosphoprotein (VASP). VASP can be phosphorylated on Ser157 (murine Ser158) in a cAMP-dependent fashion. The phosphorylation of VASP results in conformational changes of the cellular surface, and is an important component of inhibitory pathways. Vasodilator-stimulated phosphoprotein phosphorylation also influences the presence of the CD11b receptor on neutrophils and of GP IIb/IIIa on platelets. Screening for VASP phosphorylation is used clinically as efficiency control for aggregation inhibitors (clopidogrel) and is known as the platelet reactivity index. Given the importance of PNCs for the extent of inflammatory organ injury and the fact that VASP might affect the formation of PNCs, we pursued the role of VASP on the formation of PNCs and the functional impact of this during myocardial IR injury. As directed by in vitro data demonstrating a significant role of VASP for PNC formation, we found a significant reduction of intravascular PNC formation and reduced presence of PNCs within ischemic myocardium in VASP−/− mice. Using bone marrow chimeric animals, we were able to identify that phosphorylation of hematopoietic VASP is an important determinant of PNC formation and for this reason affects the degree of myocardial IR injury.

**Methods**

**Mice**

All animal protocols were in accordance with the German guidelines for use of living animals and were approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen. Vasodilator-stimulated phosphoprotein–deficient mice were generated, validated, and characterized as described previously. The wild-type (WT) controls (C57BL/6J mice) were bred as littersmates of VASP−/− mice and selected to be similar in age, gender, and weight.

**Murine Model of Myocardial Ischemia**

After anesthesia was induced, animals were placed on a temperature-controlled and heated table to maintain body temperature at 37°C. Animals were orally intubated and ventilated (Servo 900C, Siemens, Germany). After left parasternal thoracotomy, the left coronary artery was visually identified and an 8.0 nylon suture (Prolene, Ethicon, Norderstedt, Germany) was placed around the vessel. Ischemia was induced using a model described previously. Infarct sizes were determined by calculating the percentage of myocardial infarction compared to the area at risk (AAR) using double staining technique with Evan blue and triphenyltetrazolium chloride. Area at risk and the infarct size were determined via planimetry using National Institutes of Health software Image 1.0, and the degree of myocardial damage was calculated as a percentage of infarcted myocardium from the AAR.

**Pharmacological Compounds Used**

Prostaglandin E1 (PGE1) (0.14 μg · kg−1 · h−1), Sigma-Aldrich, Munich, Germany), atrial natriuretic peptide (ANP) (0.04 μg · kg−1 · h−1), Sigma-Aldrich) or vehicle (0.9% NaCl) was administered by intra-arterial infusion beginning 5 minutes before reperfusion.

**In Vivo Small Interfering RNA Repression**

To achieve in vivo repression of VASP, we used 2.2 μg/g body weight VASP ON-TARGETplus SMARTpool Mouse small interfering RNA (siRNA) dissolved in 5% Glucose- solution. As control, nontargeting siRNA (Thermo Scientific, Lafayette, CO), with at least 4 mismatches to any human, murine, or rat gene, was used. The siRNA target sequences of the VASP pool were J-046659 to 17 with target sequence UGA CAU UGC UGG AGCCAA A, J-046659 to 18 with target sequence AGG AAA UCA UCG AAG UCU U, J-046659 to 19 with target sequence GGG CUA CUG UGC UUU A, J-046659 to 20 with target sequence GAG CUG AGG CGG GGU U.

**In Vivo Depletion of Murine Neutrophils and Platelets**

We achieved neutrophil depletion using neutrophil-specific antibody treatment (RB6-8C5, 150 μg per mouse, intraperitoneally; BD Bioscience) 24 hours before the experiments. We achieved platelet depletion using an antibody-to-mouse thrombocyte serum (50 μl per mouse, intravenously; Accurate Chemical and Scientific) 2 hours before the experiments.

**Platelet Crossover Injection**

Platelets were separated as described previously. Platelets were counted and 1 200 000 platelets per mouse were injected into the carotid artery at the start of the surgical procedure (300 μL volume). Before injection platelets were tested through incubation with luciferase (Chromolune) to detect activation.

**Isolation of Human Neutrophils and Transendothelial Migration Assay**

Peripheral blood was taken from healthy donors into a Sarstedt Monevette containing sodium citrate (Sarstedt, Nümbrecht). Neutrophils were isolated as described previously. Platelet isolation from anticoagulated blood was performed as described previously. Migration assays were performed in the absence or presence of platelets. Briefly, 5×10^5 neutrophils were added together with 2.5×10^5 platelets to the upper chamber of transwell inserts (Costar, Germany) in which human microvascular endothelial cell monolayers were seeded on the apical aspect. A chemotactic gradient was established by adding 1 μmol/L phorbol 12-myristate 13-acetate (PMA) to the lower chambers. Transmigration studies were performed in the presence of CD11b antibody (Pharmlingen, Heidelberg, Germany) or GP IIb/IIIa antagonist ReoPro. In a subset of experiments, neutrophils and platelets were preincubated with PGE1, or ANP to study the impact of VASP phosphorylation on translocation. The number of neutrophils in the lower compartment was determined by quantification of the enzymatic activity of the azurophilic neutrophil granule protein myeloperoxidase, sampling 50 μL of the basal compartment. The generation of a standard curve by serial dilution of neutrophils was used to evaluate the cell number in the basolateral compartment. In a subset of experiments, staining of cells in the basolateral compartment was performed using Pappenheim stain according to standard protocol.

**Flow Cytometry**

Briefly, a single-cell suspension of 1×10^6 neutrophils or platelets in 100 μL of PBS was incubated with saturating concentrations of indicated primary antibodies for 20 minutes. After 3 washes, the cells were centrifuged at 200g for 5 minutes and resuspended in Cellfix. Fluorescence was evaluated on a FACSAir2 after stimulation with PMA, and data were analyzed by using Cell Quest software (all BD Biosciences, Germany). Antibodies used were CD41a, GP IIb/IIIa epitope PAC-1 (both BD Biosciences), anti-CD18 and anti-CD11b (both BD Bioscience, Germany). In a subset of experiments, neutrophils and platelets were preincubated with PGE1, or ANP to study the impact of VASP phosphorylation.
For murine flow cytometry, blood was gently collected by cardiac puncture and was immediately anticoagulated. Citrated whole blood samples were incubated with anti-CD42b-PE antibody (Ab) (EMFRET Analytics, Germany) and optionally anti-CD15-FITC Ab (EMFRET Analytics) or anti-CD45-FITC Ab (BD Biosciences) or anti-CD62P-FITC Ab (SantaCruz, Biotechnology, CA). After 30 minutes incubation at 37°C, samples were fixed using CellFix (BD Biosciences). Flow cytometric analysis was performed on a FACScan cytomter (BD Biosciences) as described previously. Platelet-neutrophil aggregates were detected according to the fluorescence of the anti-CD15-FITC Ab and anti-CD45-FITC Ab. In all experiments, suitable isotype controls were used to adjust for nonspecific antibody binding.

Western Blots for VASP and VASP Phosphorylation
Thrombocytes were stimulated with either 10 μM ANP or 10 μM PGE1 and lysed. After spinning at 14 000 g for 10 minutes to remove cell debris, the pellet was resuspended in RIPA buffer, and protein concentration was measured. Primary antibodies were for VASP (Cell Signaling, distributed by New England Biolabs, Frankfurt am Main, Germany), pVASP157 (Cell Signaling), and pVASP239 (Cell Signaling). Loading conditions were controlled by staining human β-actin using a murine monoclonal antibody (Cell Signaling). Murine Western blot analysis was performed as described above.

Immunohistochemistry of Neutrophils and Platelets in Murine Tissue
Immunohistochemical staining was performed with Vectastain ABC Kit (Linaris, Wertheim, Germany). After inhibiting the nonspecific binding sites with Avidin blocking solution (Vector, Burlingame, CA), the sections were incubated with primary antibody (rabbit antimouse CD41, Abcam) overnight at 4°C. Tissue sections were then incubated with biotinylated anti rabbit immunoglobulin for 1 hour followed by Vectastain ABC Reagent for 30 minutes, and then developed with a DAB substrate. For neutrophil staining, the procedure was repeated using rat antimouse neutrophil antibody (AbD Serotec, Düsseldorf, Germany) and histogreen as substrate (Linaris). Counterstaining was performed using nuclear fast red (Linaris). Histological sections were evaluated for the presence of PNCs by manual count within 3 independent tissue sections of each animal.

Generation of Bone Marrow Chimeras
In short, male donor mice (8 to 10 weeks old, 20 to 25 g) were euthanized, marrow harvested by flushing the marrow cavity and bone marrow cells were then centrifuged at 400 g for 5 minutes, resuspended and counted. Recipient mice (8 to 10 weeks of age, 20 to 25 g) were irradiated with a total dose of 12 Gy from a 137Cs source. Immediately after irradiation, 10⁷ BM cells/recipient were injected in 0.9% sodium chloride into the tail vein. The resulting chimeric mice were housed in microisolators for at least 8 weeks before experimentation and fed with water containing tetracycline (100 mg/L) in the first 2 weeks after bone marrow transplantation. Bone marrow cells were transplanted to generate (1) [WT→WT], (2) [VASP⁻⁻→VASP⁻⁻] mice as controls, (3) [WT→VASP⁻⁻], and (4) [VASP⁻⁻→WT] chimeric mice.

Troponin I Measurement
Blood was collected by central venous puncture for troponin I measurements using a quantitative rapid troponin I assay (Life Diagnostics Inc, West Chester, PA).

Data Analysis
All values are expressed as mean±SEM. Using the Kolmogorov-Smirnov test, we could show that the measured values were approximately normally distributed. Statistical significance was determined using 1-way ANOVA followed by the Bonferroni multiple-comparison test. Student t test was used where appropriate. A value of P<0.05 was considered significant.

Results

VASP/vasodilator-stimulated phosphoprotein (VASP) phosphorylation affects neutrophil facilitated transendothelial platelet movement

The importance of PNC formation for inflammatory tissue injury was reported previously. In an initial experiment, we performed transendothelial movement studies to transfer previous findings reported in an epithelial cell model. Using a chemotactic gradient across an endothelial monolayer, we found that neutrophils readily migrate across endothelial monolayers in response to phorbol 12-myristate 13acetate (PMA) stimulation (online-only Data Supplement Figure I). When exposing neutrophils and platelets in combination to this experiment, we found a robust increase of cell number and of ATP content within the basolateral compartment (Figure 1A and 1B and online-only Data Supplement Figure II). We then preincubated neutrophils and platelets with a CD11b antibody or the GP IIb/IIa antagonist ReoPro before conducting transendothelial movement studies. This resulted in a significant decrease of cell number and ATP content in the basolateral compartment (Figure 1A and 1B).

Previous work has suggested that actin dynamics might influence the exposure of CD11b/CD18 or GP IIb/IIa on the cellular surface. A crucial regulator of actin dynamics in VASP, and phosphorylation of VASP rapidly mediates platelet shape changes. Vasodilator-stimulated phosphoprotein contains 3 possible phosphorylation sites (Ser157, Ser239, and Thr278), and 2 of these are described as important determinants of platelet function (Ser157 and Ser239). We therefore proceeded to selectively phosphorylate the VASP Ser157 site (through PGE1) and the VASP Ser239 site (through ANP) to pursue the role of this on PNC formation and transendothelial PNC movement (Figure 1C). After preincubation with PGE1 or ANP, we found a significant reduction of cell number and ATP concentration within the basolateral compartment (Figure 1D and 1E). As a consequence of transendothelial movement, large PNC complexes were present in the basolateral compartment in control experiments but not after preincubation of neutrophils and platelets with PGE1 or ANP (Figure 1F and online-only Data Supplement Figure III).

Next, we investigated whether GP IIb/IIa expression and activation, as well as CD11b expression, would be affected by PGE1 or ANP treatment. Prostaglandin E1 or ANP treatment had no influence on the global expression of the GP IIb/IIa complex on platelets. However, the induction of the high-affinity binding state of GP IIb/IIa was significantly reduced after incubation with PGE1 or ANP. We did not observe alterations of CD11b expression on the surface of neutrophils by PGE1 or ANP treatment (Figure 1G and 1H and online-only Data Supplement Figure III).
Figure 1. Vasodilator-stimulated phosphoprotein phosphorylation reduces PNC formation and neutrophil-facilitated transendothelial platelet movement. Neutrophils ($5 \times 10^5$) and platelets ($2.5 \times 10^7$) were incubated in the presence or absence of either CD11b antibody or GP IIb/IIIa antagonist (ReoPro). Phorbol 12-myristate 13-acetate (1 μmol/L) was present in the basolateral compartment to induce a chemotactic gradient. Translocation across human microvascular endothelial cells was determined by the presence of cell count or ATP concentration in the basolateral compartment after 60 minutes. A, Cell number in the basolateral compartment. B, ATP content in the basolateral compartment. C, Vasodilator-stimulated phosphoprotein Ser$^{157}$ phosphorylation induced through PGE$_1$ and VASP Ser$^{239}$ phosphorylation through ANP in the platelet-neutrophil combination before transmigration studies. D, Cell number in the basolateral compartment after preincubation with either PGE$_1$ or ANP. E, ATP content in the basolateral compartment after preincubation with either PGE$_1$ or ANP. F, Representative cytospin samples obtained from the basolateral compartment demonstrating PNCs in the absence or presence of PGE$_1$ or ANP. G, Expression of CD41a or PAC-1 on the surface of platelets preincubated with PGE$_1$ and stimulated with PMA determined by flow cytometry. H, Expression of CD41a or PAC-1 on the surface of platelets preincubated with ANP and stimulated with PMA determined by flow cytometry (Data are shown as mean±SEM; n=12 to 16 per group for transendothelial studies, n=4 for cytospin; *P<0.05; **P<0.01; ***P<0.001 as indicated). PMA indicates phorbol 12-myristate 13-acetate; VASP, vasodilator-stimulated phosphoprotein; ANP, atrial natriuretic peptide; PGE$_1$, prostaglandin E$_1$; and PNC, platelet-neutrophil complex.
**Platelet Depletion, Neutrophil Depletion, and Crossover Injection Identify the Importance of Platelet-Neutrophil Complexes for Myocardial Ischemia-Reperfusion Injury**

To gain further insight whether the observed protective effect of VASP depletion is indeed mediated through PNCs, we selectively depleted platelets before animals were exposed to the model of myocardial IR injury. Platelet depletion resulted in a significant reduction of myocardial IR injury infarct size in WT and in VASP$^{-/-}$ animals. Wild-type animals demonstrated an infarct size of 4.5±2.4%. VASP$^{-/-}$ animals of 3.5±1.4% (Figure 3A through 3C). When examining tissue sections after myocardial IR injury, we did not find PNCs within the tissue sections of WT or VASP$^{-/-}$ animals (Figure 3D and 3E). Depletion of neutrophils resulted in a similar finding: Myocardial infarct size was significantly reduced in WT animals (5.4±1.4%) and in VASP$^{-/-}$ animals (5.3±1.3%) (Figure 3F through 3H). Platelet-neutrophil complexes were not present within myocardial tissue, corroborating the importance of PNC formation for myocardial IR injury.

Next, we asked whether PNC formation and myocardial IR injury would be influenced by VASP expression of platelets. We separated platelets from WT or VASP$^{-/-}$ animals and performed crossover injection of these platelets preceding IR injury. The separation process we used did not result in an activation of the extracted platelets, and separated platelets were still functionally sound (online-only Data Supplement Figure VIII). We then transferred VASP$^{-/-}$ platelets into WT animals before the start of myocardial ischemia and found that this resulted in a reduction of myocardial IR injury of ~40% compared with WT-to-WT injected animals (Figure 4A through 4C). In contrast, VASP$^{-/-}$ animals injected with WT platelets demonstrated an increase in infarct size. The reduced size

**Figure 2. VASP-deficient animals demonstrate reduced PNC formation and attenuated myocardial IR injury.** A, Infarct size in VASP$^{-/-}$ mice compared with WT mice after 60 minutes of myocardial ischemia followed by 2 hours reperfusion. Calculated is the percentage of necrotic tissue to AAR. B, Correlating serum troponin I measurement of VASP$^{-/-}$ and WT animals. C, Representative images of myocardial infarcts (blue/dark=retrograde Evan blue staining; red and white=AAR; white=infarcted tissue). D, Number of PNCs detected in whole blood 5 minutes after the initiation of reperfusion in WT and VASP$^{-/-}$ mice. E, Representative histological images of PNCs (neutrophil=blue; platelet=black) in tissue sections of myocardial infarct of VASP$^{-/-}$ and WT animals. F, Number of PNCs present in histological sections of VASP$^{-/-}$ and WT animals at the end of the study period (data are shown as mean±SEM; n=6; **P<0.01; ***P<0.001 as indicated; tissue sections magnification ×400; insert ×1000). AAR indicates area at risk; WT, wild type; VASP, Vasodilator-stimulated phosphoprotein; and PNCs, platelet-neutrophil complexes.
of myocardial tissue damage was reflected in serum troponin I levels (Figure 4B). When determining the PNC count within tissue sections, we found a reduced presence of PNCs within tissue of WT animals receiving VASP<sup>−/−</sup> platelets and an increased presence of PNCs in VASP<sup>−/−</sup> animals injected with WT platelets within the myocardial tissue (Figure 4E).

**Platelet-Neutrophil Complexes Formation Is Dependent on Hematopoietic Vasodilator-Stimulated Phosphoprotein Expression**

To further clarify the role of VASP in myocardial IR injury and the formation of PNCs, we generated chimeric animals using bone marrow transplantation (online-only Data Supplement Figure IX). Six weeks after bone marrow transplantation, we performed the above-described myocardial IR model. In chimeric animals, myocardial IR injury was dependent on hematopoietic VASP expression, demonstrating reduced myocardial damage in the VASP<sup>−/−</sup>→WT transplanted animals. The control transplanted animals (WT→WT and VASP<sup>−/−</sup>→VASP<sup>−/−</sup>) reflected the results of the WT or VASP<sup>−/−</sup> animals (Figure 5A through 5C). We then determined the presence of PNCs within the blood 5 minutes after the initiation of reperfusion and found that the WT→VASP<sup>−/−</sup> transplanted animals demonstrated a similar number of PNCs within the blood as the WT→WT transplanted animals did. In the VASP<sup>−/−</sup>→WT transplanted animals, we found a significantly reduced number of PNCs within the blood compared to the WT→VASP<sup>−/−</sup> transplanted animals (Figure 5D). When examining myocardial tissue sections for the presence of PNCs, we found that VASP<sup>−/−</sup>→WT transplanted animals showed a reduced presence of PNCs compared with the WT→VASP<sup>−/−</sup> transplanted animals. This was associated with a decrease in myocardial tissue destruction (Figure 5E and 5F).

Figure 3. Platelet or neutrophil depletion prevents PNC formation and attenuates myocardial IR injury in WT and VASP<sup>−/−</sup> mice. A, Myocardial IR injury in WT and VASP<sup>−/−</sup> animals after platelet depletion. Infarct sizes were measured by double staining with Evan blue and triphenyltetrazolium chloride after 2 hours of reperfusion and (B) correlating serum troponin I values. C, Representative images of myocardial sections of infarcts after platelet depletion (blue/dark, retrograde Evan blue staining; red and white, infarcted tissue). D, Platelet-neutrophil complexes in WT and VASP<sup>−/−</sup> animals after platelet depletion (neutrophil=blue; platelet=black). E, Number of PNCs present in histological sections of VASP<sup>−/−</sup> and WT animals after platelet depletion. F, Myocardial ischemia in WT and VASP<sup>−/−</sup> animals after neutrophil depletion and IR. Infarct sizes were measured by double staining with Evan blue and triphenyltetrazolium chloride after 60 minutes of ischemia and 2 hours of reperfusion and correlating (G) serum troponin I values. H, Representative images of myocardial sections of infarcts after neutrophil depletion (blue/dark, retrograde Evan blue staining; red and white, AAR; white, infarcted tissue). I, Platelet-neutrophil complexes in WT and VASP<sup>−/−</sup> animals after neutrophil depletion, 60 minutes of ischemia, and 2 hours of reperfusion. J, Number of PNCs present in histological sections of VASP<sup>−/−</sup> and WT animals after neutrophil depletion (data are shown as mean±SEM; n=6; tissue sections magnification ×400; insert ×1000). WT indicates wild type; VASP, vasodilator-stimulated phosphoprotein; AAR, area at risk; and PNCs, platelet-neutrophil complexes.
Phosphorylation of Hematopoietic Vasodilator-Stimulated Phosphoprotein Reduces Platelet-Neutrophil Complexes Formation and Myocardial Ischemia-Reperfusion Injury

Our previous experiments have demonstrated that hematopoietic-derived VASP expression is of crucial importance for formation of PNCs during myocardial IR injury. To provide more evidence for this, we continued to test the role of VASP phosphorylation in chimeric animals. For this purpose, we employed PGE$_1$ or ANP in VASP$^{-/-}$→WT and WT→VASP$^{-/-}$ transplanted animals. Evaluation of the infarct size revealed that PGE$_1$ significantly reduced myocardial tissue damage in the WT→VASP$^{-/-}$ transplanted animals but had no effect in the VASP$^{-/-}$→WT transplanted animals. Similarly, ANP resulted in a significant reduction of myocardial tissue damage through VASP phosphorylation in the WT→VASP$^{-/-}$ transplanted animals, but had no effect in the WT→VASP$^{-/-}$ transplanted animals. Troponin I measurement confirmed this finding (Figure 7A through 7C). When examining the presence of PNCs within the blood of these animals, we found that the infusion of PGE$_1$ or ANP resulted in a significant reduction of PNCs in the blood of WT but not of VASP$^{-/-}$ animals (Figure 6E). We then proceeded to identify the potential presence of PNCs in myocardial tissue and found that WT animals treated with PGE$_1$ or ANP demonstrated a reduced number of PNCs in myocardial tissue compared with controls. Platelet-neutrophil complexes were not reduced in VASP$^{-/-}$ animals after PGE$_1$ or ANP exposure (Figure 6F and 6G).

Discussion

Recent work has appreciated the importance of PNC formation during acute lung injury and has demonstrated that neutrophils facilitate the transepithelial movement of platelets. The role of PNCs during IR injury to date is however not known. We demonstrate here that the formation of PNCs...
has a significant impact on the extent of myocardial IR injury and that phosphorylation of the cytoskeletal protein VASP affects the formation of PNCs. In initial in vitro studies, we found that neutrophils facilitate the movement of platelets across an endothelial monolayer and that this process can be attenuated through phosphorylation of VASP. Studies employing VASP−/− and chimeric animals revealed that hematopoietic VASP significantly influences the formation of PNCs in vivo. Furthermore, VASP phosphorylation dampened the intravascular formation during reperfusion and the movement of PNCs into ischemic myocardial tissue and for this reason significantly reduced myocardial IR injury. Therefore, these studies define the role of PNCs for myocardial tissue injury and identify VASP as a key regulator of PNC formation.

The formation of PNCs is achieved through fibrinogen bridging between the GP IIb/IIIa receptor on platelets and CD11b/CD18 on neutrophils. In in vitro studies we found that the expression of GP IIb/IIIa on platelets is altered through the phosphorylation of VASP on Ser157 and Ser239. We used ANP to selectively induce phosphorylation of VASP at Ser239 and PGE1 to induce VASP phosphorylation at Ser157. This resulted in a reduction of PNC formation in vitro and in vivo. This finding is supported by a study from Scotland et al in which platelet-leukocyte interactions and PNC formation were reduced through C-type natriuretic peptide. C-type natriuretic peptide increases intracellular cGMP with a subsequent phosphorylation of VASP on Ser239. This reduction of PNC formation was accompanied by a reduced expression of P-selectin on the platelet surface. An increase of intracellular cGMP within platelets is also achieved through nitric oxide, which is a well-defined inhibitor of platelet activation and P-selectin expression. The cAMP-dependent phosphorylation of VASP at Ser157 reduces the affinity of the platelet fibrinogen receptor to bind fibrinogen and collagen. In partial contrast to this is a study by Massberg et al in which the authors demonstrate an increased tethering of VASP−/− platelets on the vascular wall. The authors concluded that the loss of VASP increased loose platelet adhesion that was dependent on P-selectin expression and that following denudation of the vascular wall increased adhesion of VASP−/− platelets was dependent on the platelet GP IIb/IIIa receptor. But the authors also point out that VASP expression within the vascular wall is essential to control platelet-endothelial interactions. The primary focus of our study however was not on the tethering of platelets on the vascular wall but rather on the firm linkage between neutrophils and platelets for PNC formation. Therefore, given the previously discussed literature, the finding that VASP phosphorylation through ANP or PGE1 alters PNC formation might well explain...
the reduction of PNC formation. This would at least in part provide further explanation to the clinically observed protective role of ANP and PGE1 during myocardial IR injury.36,37

The functional importance of PNC formation for the extent of inflammatory tissue injury has been implicated previously. Kupatt et al demonstrated that a reduction of PNC formation through c7E3Fab, a chimeric Fab fragment blocking the GP IIb/IIIa receptor resulted in improved postischemic recovery of the external heart.38 The importance of the formation of PNCs for the extent of inflammatory tissue damage has been recognized in a recent study by Zarbock et al examining the role of the PNCs during acute lung injury.9 Zarbock et al demonstrated that acid-induced lung injury is aggravated by the formation of PNCs. The inhibition of this PNC formation resulted in a marked decrease in pulmonary injury and improved pulmonary function. A subsequent study by Loo-
ney et al corroborated this by demonstrating that platelet depletion or aspirin pretreatment resulted in reduced transfusion-related lung injury. In this study, the interaction of neutrophils with platelets was not dependent on P-selectin expression.39 Weissmüller et al demonstrated that after formation PNCs are translocated across an epithelial cell barrier. The translocated platelets subsequently released ATP, which induces the function of ectonucleotidases in the intestinal lumen.10,40 This demonstrates that translocation of PNCs across a cellular layer is possible and that the translocated platelets are functionally intact. In line with these previous studies, we demonstrate here that neutrophils facilitate the transendothelial movement of platelets forming PNCs in vitro and that this PNC formation and movement is also present in vivo. Furthermore, we demonstrate that neutrophils-facilitated platelet movement can be reduced through the phosphorylation of VASP, which has a significant impact on myocardial IR injury. This finding correlates well with the clinical strategy to reduce platelet activation during the reperfusion phase.41 The selective depletion of platelets or neutrophils we used here demonstrates that the formation of PNCs is of crucial importance for the extent of myocardial IR injury.

In summary, the results of our study are in line with previous investigations demonstrating the importance of PNCs for the extent of inflammatory tissue injury and confirm that the reduction of PNC formation might be tissue protective. The regulatory role of VASP phosphorylation for the formation of PNC was identified in vitro and transferred into in vivo evidence. Vasodilator-stimulated phosphoprotein phosphorylation resulted in reduced formation of PNC and dampened the extent of myocardial IR injury. This work therefore increases our understanding of the mechanisms underlying the formation of PNCs, supports the importance of PNCs for the extent of IR injury, and increases our understanding of the role of VASP during myocardial IR injury.

Acknowledgments

We thank Stephanie Zug for help with the immunohistochemistry assay and Stefanie Laucher for help with the performance of animal experiments. We thank Peter Mayer for technical assistance obtaining the figures of the myocardial tissue sections. The authors acknowledge that, given the large number of statistical comparisons made, the possibility of a type I error must be taken into consideration.
Sources of Funding
This work was supported by Deutsche Forschungsgemeinschaft (DFG) grant DFG-RO 3671/4-1 to Dr Rosenberger, by a grant from the Karl Kuhn Foundation for Cardiovascular Research to Dr Rosenberger, by Sonderforschungsbereich SFB688/ TP A2 to Dr Walter, and by the Sonderforschungsbereich SFB-815 (project 17) to Dr Zacharowski.

Disclosures
None.

References


**CLINICAL PERSPECTIVE**

The formation of platelet-neutrophil complexes has significant impact on the extent of inflammatory and ischemic tissue injury. In the present study, we demonstrate that vasodilator-stimulated phosphoprotein, a crucial cytoskeletal protein involved in platelet activation, influences the formation of these platelet-neutrophil complexes. The phosphorylation of vasodilator-stimulated phosphoprotein during the reperfusion phase, achieved through the administration of prostaglandin E1 or atrial natriuretic peptide, significantly attenuates the formation of platelet-neutrophil complexes and as a result reduces the extent of myocardial ischemia-reperfusion injury. This implies that phosphorylation of vasodilator-stimulated phosphoprotein during reperfusion has cardioprotective potential and could be pursued as a future strategy to reduce myocardial reperfusion injury.
Phosphorylation of Vasodilator-Stimulated Phosphoprotein Prevents Platelet-Neutrophil Complex Formation and Dampens Myocardial Ischemia-Reperfusion Injury
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_Circulation_. 2011;123:2579-2590; originally published online May 23, 2011;
doi: 10.1161/CIRCULATIONAHA.110.014555
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL
Phosphorylation of vasodilator-stimulated phosphoprotein (VASP) prevents platelet-neutrophil complex formation and dampens myocardial ischemia-reperfusion injury.

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Supplemental Figure 2
Supplemental Figure 4

Fluorescence Intensity

CD18  CD11b (active)

- neg
- neg +ANP
- neg +PGE1
- PMA
- PMA +ANP
- PMA +PGE1

Supplemental Figure 4
Supplemental Figure 6
Supplemental Figure 8
<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>VASP-/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>anaesthesia (mmHg)</td>
<td>95±2</td>
<td>93±2</td>
</tr>
<tr>
<td>open chest (mmHg)</td>
<td>83±2</td>
<td>88±2</td>
</tr>
<tr>
<td>ischemia (mmHg)</td>
<td>75±2</td>
<td>83±2</td>
</tr>
<tr>
<td>reperfusion (mmHG)</td>
<td>77±1</td>
<td>77±3</td>
</tr>
<tr>
<td>reperfusion + PGE1 (mmHG)</td>
<td>72±1</td>
<td>76±2</td>
</tr>
<tr>
<td>reperfusion + ANP (mmHG)</td>
<td>68±6</td>
<td>73±2</td>
</tr>
</tbody>
</table>
Supplemental Figure 11

A

Infarct Size [% AAR]

+PGE1 +ANP

0 10 20 30 40 50 60

*** ***

B

Troponin [ng/ml]

+PGE1 +ANP

0 20 40 60 80 100 120

*** ***

C

+ PGE1

WT → WT VASP−/− → VASP−/−

D

+ ANP

WT → WT VASP−/− → VASP−/−

E

WT → WT VASP−/− → VASP−/−

F

WT → WT VASP−/− → VASP−/−
Supplemental Figure Legends

**Supplemental Figure 1.** Chemotactic gradient generated through PMA. Neutrophils (5x10^5) were incubated in the presence or absence of 1 μM Phorbol 12-myristate 13-acetate (PMA) in the apical and/or basal chamber. Translocation across endothelial HMEC-1 was determined by the presence of cell count in the basolateral compartment after 60 minutes (Results are obtained from 4 monolayers in each condition; Data are Mean ± SEM).

**Supplemental Figure 2.** Passive movement of platelets, neutrophils or both across endothelial monolayers. Neutrophils (5x10^5) and platelets (2,5x10^7) were incubated in the presence or absence of either CD11b antibody or GPIIb/IIIa antagonist (ReoPro®) in the apical compartment. Translocation across endothelial HMEC-1 was determined in the absence of Phorbol 12-myristate 13-acetate (PMA) in the basolateral compartment after 60 minutes. A) Cell number in the basolateral compartment B) ATP content in the basolateral compartment (Results are obtained from 6 monolayers in each condition; Data are Mean ± SEM).

**Supplemental Figure 3.** Representative papenheim stainings of neutrophils and platelets. Pictures taken at x400 and x1000 magnification.

**Supplemental Figure 4.** Expression of CD18 and CD11b on the surface of neutrophils in response to PMA. Fluorescence expression of CD18 and CD11b on the surface of PMN pre-incubated with PGE1 or ANP prior to stimulation with PMA (Data are Mean ± SEM, n=3).
Supplemental Figure 5. Characterization of platelets form WT and VASP<sup>−/−</sup> animals. A) Expression of the GP IIb/IIIa receptor and B) P-selectin on the surface of platelets of WT and VASP<sup>−/−</sup> animals. (Data are Mean ± SEM, n=4)

Supplemental Figure 6. VASP expression in WT animals following siRNA injection. Western blot analysis of WT animals 24h post siVASP or siSCR injection (Pooled samples of n=4/group).

Supplemental Figure 7. In-vivo VASP repression by siRNA dampens myocardial IR injury and prevents PNC formation. A) Myocardial IR Injury after in-vivo targeted repression of VASP with siRNA (siVASP) or non-targeting siRNA (siSCR) and B) Correlating serum troponin I values C) Representative images of myocardial infarcts (blue/dark= retrograde Evan’s blue staining; red and white= area at risk; white= infarcted tissue). D) Histological images of platelet-neutrophil complexes (PNC) (neutrophil = blue; platelet = black) in tissue sections of myocardial infarct of siSCR or siVASP treated WT animals after IR E) Mean number of PNCs present in histological sections of siSCR or siVASP treated WT animals at the end of the study period (Data are shown as Mean ± SEM, n=6, **P < 0.01; ***P < 0.001 as indicated, tissue sections magnification x400 and x1000, n=5).

Supplemental Figure 8. Platelet activity following platelet separation. After separation, platelets were tested for activation using luciferase dependent ATP measurement A) Platelets prior to injection B) Control following stimulation with thrombin to demonstrate that platelets can be activated (All Data are Mean ± SEM, n=8, ***P < 0.001 as indicated)
**Supplemental Figure 9.** VASP expression in chimeric animals. Western blot analysis of chimeric animals following bone marrow transplantation demonstrating VASP expression in the myocardium and blood of WT→WT transplanted animals, myeloid WT into $VASP^{+/−}$ animals (WT→$VASP^{+/−}$), myeloid $VASP^{+/−}$ in WT animals ($VASP^{+/−}$→WT), and $VASP^{+/−}$→$VASP^{+/−}$ transplanted control animals (Pooled samples of n=4/group).

**Supplemental Figure 10.** Hemodynamic values during experimental protocol. Experimental animals were cannulated with a catheter into the carotid artery and blood pressure measurements determined during ischemia, reperfusion, injection of atrial natriuretic peptide (ANP) or prostaglandin E1 (PGE1) (All Data are Mean ± SEM, n=8)

**Supplemental Figure 11.** Myocardial ischemia-reperfusion injury in WT→WT and $VASP^{+/−}$→$VASP^{+/−}$ transplanted animals treated with prostaglandin E1 (PGE1) or atrial natriuretic peptide (ANP) during reperfusion. A) Size of myocardial IR injury in WT→WT and $VASP^{+/−}$→$VASP^{+/−}$ animals after 60 min of myocardial ischemia following 2 hours reperfusion and infusion of vehicle, PGE1 or ANP. Calculated is the percentage of necrotic tissue to the area at risk (AAR). B) Correlating serum troponin I measurement C) Representative images of myocardial sections of infarcts from the experiment in above after infusion of PGE1 or D) ANP (blue/dark, retrograde Evan’s blue staining; red and white, area at risk; white, infarcted tissue) E) Platelet-neutrophil complexes (PMNs = blue; platelets = black) in sections of myocardial infarct tissue of chimeric animals (WT→WT and $VASP^{+/−}$→$VASP^{+/−}$ animals) after 60 min of myocardial ischemia following 2 hours reperfusion and infusion of PGE1 or F) ANP during reperfusion (Data are shown as Mean ± SEM, n=4,
***P < 0.001 as indicated, tissue sections magnification x400 and x1000, one representative individual experiment is demonstrated).