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−/− 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 the presence of PNCs within ischemic myocardial tissue and the extent of myocardial IR injury. Furthermore, phosphorylation of VASP on Ser153 or Ser235 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

Approximately 3.8 million men and 3.4 million women die of myocardial ischemia-reperfusion (IR) injury each year.1 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.2 This structural derangement is caused in part by neutrophils, which are attracted into the ischemic tissue.3-5 Platelets also participate in the process of neutrophil recruitment and aggravate myocardial tissue damage during the reperfusion phase.6,7 Finally, these events result in an inflammatory response, which has significant impact on apoptotic events within the myocardium.8

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.9,10 In a study by Zarbock et al, the extent of acute lung injury was significantly reduced by blocking the formation of PNCs.9 Furthermore, Weismueler et al demonstrated that neutrophils facilitate transepithelial movement of platelets into the intestinal lumen.10 Firm adhesion between neutrophils and platelets is achieved by fibrinogen binding to CD11b/CD18 on neutrophils and the glycoprotein (GP) IIb/IIIa receptor on platelets.

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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 Ser159) in a cAMP and on Ser205 (murine Ser207) in a cGMP-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 littermates 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 at the start of the surgical procedure (300 μL volume). Before injection platelets were tested through incubation with luciferase (Chronolume) to detect activation.

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 FACs Calibur 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 on translocation.
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) (EMD Millipore, Reading, UK) and optionally anti-CD15-FITC Ab (EMD Millipore) 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 FACScan cytometer (BD Biosciences) as described previously.27 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 Merck Biosciences, Darmstadt, Germany), pVASP (Ser157) (Cell Signaling), and pVASP (Ser239) (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 (Vector Laboratories, Burlingame, CA). Tissues 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 and sites with Avidin blocking solution (Vector, Burlingame, CA), and histogreen as substrate (Linaris). 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 Merck Biosciences, Darmstadt, Germany), pVASP (Ser157) (Cell Signaling), and pVASP (Ser239) (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.

Results

Vasodilator-Stimulated Phosphoprotein Phosphorylation Affects Neutrophil Facilitated Transendothelial Platelet Movement
The importance of PNC formation for inflammatory tissue injury was reported previously.5,9 In an initial experiment, we performed transendothelial movement studies to transfer previous findings reported in an epithelial cell model.10 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/IIIa 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 expression of CD11b/CD18 or GP IIb/IIIa on the cellular surface.19,28 A crucial regulator of actin dynamics is VASP, and phosphorylation of VASP rapidly mediates platelet shape changes.29 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/IIIa 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/IIIa complex on platelets. However, the induction of the high-affinity binding state of GP IIb/IIIa 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 x 10^6) and platelets (2.5 x 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.
Vasodilator-Stimulated Phosphoprotein−/− Mice Demonstrate Reduced Platelet-Neutrophil Complexes Formation and Attenuated Myocardial IR Injury

We then proceeded to investigate whether this finding could have in vivo significance and used a model myocardial IR injury for this. Initially, we determined whether VASP−/− animals would demonstrate altered characteristics of receptor expression. We determined the expression of GP IIb/IIIa and P-selectin, but did not find a difference between WT or VASP−/− animals (online-only Data Supplement Figure V). We then exposed VASP−/− mice and littermate controls to 60 minutes of myocardial ischemia followed by 3 hours of reperfusion. VASP−/− deficient mice demonstrated a significant reduction of myocardial infarct size compared with controls; this was corroborated through serum troponin I levels (Figure 2A through 2C). Furthermore, 5 minutes after the initiation of reperfusion, the number of PNCs in whole blood samples was significantly lower in VASP−/− animals compared with WT controls (Figure 2D). At the end of the reperfusion period, we obtained tissue sections of the affected myocardium and examined these sections for the presence of PNCs. We found a reduced presence of PNCs within tissue of VASP−/− animals compared with littermate controls (Figure 2E and 2F).

To gain further insight into the specific role of VASP, we used gene-targeted repression using siRNA and injected animals 24 hours before the start of the experiment with siVASP or control siRNA. This resulted in significant VASP repression in vivo (online-only Data Supplement Figure VI). We then proceeded to subject these animals to the myocardial IR model and found reduced myocardial tissue damage in the siVASP treated animals compared to siRNA controls. This was associated with a reduced presence of PNCs within the affected myocardial tissue (online-only Data Supplement Figure VII).

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 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
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).
Vasodilator-Stimulated Phosphoprotein Phosphorylation Reduces In Vivo Platelet-Neutrophil Complexes Formation and Myocardial IR Injury

We were able to demonstrate the in vitro relevance of VASP phosphorylation for PNC formation and transendothelial movement of PNCs. It has been reported that in vivo VASP phosphorylation correlates with a reduced platelet activation state. We therefore next pursued the role of VASP phosphorylation in vivo for the formation of PNCs, inducing selective VASP phosphorylation with PGE1 or ANP (Figure 6A). First, we investigated potential hemodynamic changes after PGE1 or ANP injection but did not find a significant influence of these agents on hemodynamic values (online-only Data Supplement Figure X). Prostaglandin E1 infusion reduced the extent of myocardial tissue damage in the WT animals, yet we did not observe this effect in the VASP−/− animals. When using ANP to induce VASP Ser235 phosphorylation, we also found a significant reduction of myocardial tissue injury in the WT animals, yet this was not present in VASP−/− animals (Figure 6B and 6D). Troponin I measurements confirmed these (Figure 6C). When examining the presence of circulating PNCs in the blood of these mice, we found that the infusion of PGE1 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 PGE1 or ANP demonstrated a reduced number of PNCs in myocardial tissue compared with controls. Platelet-neutrophil complexes were not reduced in VASP−/− animals after PGE1 or ANP exposure (Figure 6F and 6G).

Phosphorylation of Hematopoietic Vasodilator-Stimulated Phosphoprotein Reduces Platelet-Neutrophil Complexes Formation and Myocardial IR 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 PGE1 or ANP in VASP−/−→WT and WT→VASP−/− transplanted animals. Evaluation of the infarct size revealed that PGE1 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 WT→VASP−/− transplanted animals demonstrated a significant reduction of PNCs within the blood after infusion of PGE1 or ANP. This was however not observed in the VASP−/−→WT transplanted animals (Figure 7D). We then proceeded to identify the presence of PNCs within ischemic myocardial tissue of these animals. In tissue sections we found a reduced presence of PNCs in the WT→VASP−/− transplanted animals after PGE1 or ANP infusion, confirming the role of hematopoietic VASP for the formation of PNCs (Figure 7E and 7F).

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.9,10 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 hematopoetic 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 work.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. 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. 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 reduced platelet activation during the reperfusion phase.

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

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Disclosures

None.

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cyte interaction mediated by fibrinogen: implications for myocardial reperfusion injury. 


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

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Supplemental Figure 1

Number of Cells

- PMA apical: - + -
- PMA basal: - + +
- Neutrophils: + + +
Supplemental Figure 2
Supplemental Figure 5

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Supplemental Figure 6
Supplemental Figure 8
Supplemental Figure 9

WT → WT    WT → KO    KO → WT    KO → KO
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total VASP
GAPDH
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<td>reperfusion + ANP (mmHG)</td>
<td>68±6</td>
<td>73±2</td>
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Supplemental Figure Legends

**Supplemental Figure 1.** Chemotactic gradient generated through PMA. Neutrophils (5x10⁵) 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 (5x10⁵) and platelets (2.5×10⁷) 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^/^- animals. A) Expression of the GP IIb/IIIa receptor and B) P-selectin on the surface of platelets of WT and VASP^/^- 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<sup>−/−</sup>* animals (WT→*VASP<sup>−/−</sup>*), myeloid *VASP<sup>−/−</sup>* in WT animals (*VASP<sup>−/−</sup>→WT), and *VASP<sup>−/−</sup>→*VASP<sup>−/−</sup> 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 prostanglandin E1 (PGE1) (All Data are Mean ± SEM, n=8)

**Supplemental Figure 11. Myocardial ischemia-reperfusion injury in WT→WT and VASP<sup>−/−</sup>→VASP<sup>−/−</sup> 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<sup>−/−</sup>→*VASP<sup>−/−</sup> 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<sup>−/−</sup>→*VASP<sup>−/−</sup> 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,
\[ **P < 0.001 \text{ as indicated, tissue sections magnification x400 and x1000, one representative individual experiment is demonstrated).} \]