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Phosphorylation of Blood Vessel Vasodilator-Stimulated Phosphoprotein at Serine 239 as a Functional Biochemical Marker of Endothelial Nitric Oxide/Cyclic GMP Signaling

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ABSTRACT

The endothelium-derived relaxing factors nitric oxide (NO) and prostacyclin (PGI_2) are important antithrombotic, relaxant, and antiproliferative agents of the blood vessel wall that exert their intracellular effects primarily via cGMP- and cAMP-dependent protein kinases (cGK, cAK). However, no biochemical marker for their activity in the intact blood vessel is available except for transient increases in the concentration of cGMP and cAMP. Using Western blot analysis and specific antibodies, we show here that phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) at Ser239 (P_{Ser239}-VASP) in rabbit aorta was detectable only in segments with an intact endothelium, although at least one third of VASP is contained in the remaining vascular wall. In endothelium-denuded aorta, VASP phosphorylation was increased by the NO donor sodium nitroprusside (SNP). Levels of P_{Ser239}-VASP, in the presence of endothelium and either SNP or 8-bromo-cAMP, were maximal. VASP phosphorylation elicited by 8-bromo-cAMP was inhibited significantly by the cGK inhibitor *R*p-8-Br-PET-cGMPS. Stimulated P_{Ser239}-VASP formation was fully reversible, reaching basal levels after 10 min of repeated washouts. Consistent with the important role that the NO/cGMP pathway plays in the formation of P_{Ser239}-VASP in rabbit aorta, inhibition of NO synthase by N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1 mM) or of soluble guanylyl cyclase by 1*H*-[1,2,4]oxadiazolo[3,4-a]quinoxalin-1-one (ODQ; 50 μM) almost completely abolished P_{Ser239}-VASP formation in endothelium intact blood vessels. These data suggest that vascular P_{Ser239}-VASP is primarily regulated by the NO/cGMP pathway and may thus serve as a biochemical marker for the activity state of this essential pathway in endothelial function.

Vascular endothelium releases a variety of relaxing factors including prostacyclin (PGI_2) (Bunting et al., 1976) and nitric oxide (NO) (Palmer et al., 1987). Both are involved in the acute and long-term regulation of vascular tone (Furchgott and Vanhoutte, 1989; Somlyo and Somlyo, 1994). An abnormal increase in smooth muscle tone, due to endothelial dysfunction and alterations in the production or action of these factors, has been implicated in the pathogenesis of several cardiovascular disease states, including arterial hypertension, coronary heart disease, and atherosclerosis (Somlyo and Somlyo, 1994; Kojda and Harrison, 1999).

Relaxation of vascular smooth muscle can result from either cyclooxygenase-catalyzed PGI_2 formation, receptor-mediated activation of adenylyl cyclase (AC), and increased cAMP levels or via Ca^{2+}-triggered activation of NO synthase in endothelial cells leading to activation of soluble guanylyl cyclase (sGC) and increased cGMP levels (Rasmussen et al., 1990; Murad, 1994). Vasodilators that elevate either cGMP or cAMP inhibit the contraction of vascular smooth muscle cells and the aggregation of platelets. NO- and cGMP-induced smooth muscle relaxation and platelet inhibition are primarily mediated by cGMP-dependent protein kinase (cGK) as evidenced by the absence of this response in cGK-I knock-out mice (Pfeifer et al., 1999; Hofmann et al., 2000).

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ABBREVIATIONS: NO, nitric oxide; PGI_2, prostacyclin; AC, adenylyl cyclase; sGC, soluble guanylyl cyclase; cGK, cGMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein; cAK, cAMP-dependent protein kinase; Ena, enabled; Evl, Ena-VASP-like protein; PE, phenylephrine; ODQ, 1H-[1,2,4]oxadiazolo[3,4-a]quinoxalin-1-one; DMSO, dimethyl sulfoxide; KHB, Krebs-Henseleit buffer; 8-Br-cAMP, 8-bromo-cAMP; *R*p-8-Br-PET-cGMPS, β-Phenyl-1,N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer; P_{Ser239}-VASP, VASP phosphorylated at serine 239; L-NAME, N^{ω}-nitro-L-arginine methyl ester; SNP, sodium nitroprusside; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; NOS, nitric-oxide synthase.

The functional effects of cGK are thought to be due to the phosphorylation of specific target proteins such as the 46/50-kDa vasodilator-stimulated phosphoprotein (VASP), the IP_3 receptor and associated proteins, phospholamban, rapB1, the myosin binding subunit of myosin phosphatase, and others (Siess, 1989; Eigenthaler et al., 1999; Pfeifer et al., 1999; Hofmann et al., 2000).

VASP, a well characterized substrate for both cGK and cAMP-dependent protein kinase (cAK), is expressed in many mammalian cell types and tissues including platelets and endothelial and vascular smooth muscle cells (Waldmann et al., 1987; Halbrügge and Walter, 1989; Halbrügge et al., 1990; Reinhard et al., 1992; Draijer et al., 1995). VASP belongs to a family of proline-rich proteins that also includes the *Drosophila melanogaster* protein Enabled (Ena), its mammalian ortholog Mena, and the Ena-VASP-like protein Evl (Reinhard et al., 1999). All these proteins share common Ena-VASP homology domains 1 and 2 (EVH1 and EVH2) that are separated by a proline-rich central domain of low complexity.

In various cell types, including platelets, vascular smooth muscle cells, endothelial cells, and fibroblasts, VASP is concentrated at and associated with actin microfilaments, focal adhesions, and cell-cell contacts (Reinhard et al., 1992; Draijer et al., 1995; Reinhard et al., 1999). Functionally, VASP is an important modulator of microfilaments and regulates spatially confined actin polymerization (Niebuhr et al., 1997; Reinhard et al., 1999). In platelets, VASP phosphorylation closely correlates with the inhibition of activation of the fibrinogen receptor and integrin $\alpha_{IIb}\beta_3$ (Horstrup et al., 1994), which has recently gained substantial support by the analysis of platelets from VASP-deficient mice (Aszódi et al., 1999; Hauser et al., 1999).

In the intact blood vessel, however, the precise function of VASP remains to be elucidated. Three cAK/cGK phosphorylation sites in VASP have been identified in vitro and in intact platelets: serine 157, serine 239, and threonine 278. The sites serine 157 and serine 239 are preferred by cAK and cGK, respectively (Butt et al., 1994; Abel et al., 1995; Smolenski et al., 1998). VASP phosphorylation can be monitored in two ways: phosphorylation of serine 157 alters the electrophoretic mobility of VASP from 46 to 50 kDa, which can be used to quantify phosphorylation of this site (Butt et al., 1994; Reinhard et al., 1999). Phosphorylation of serine 239 can be measured by the monoclonal antibody 16C2, which specifically detects the phosphorylation state of this site (Smolenski et al., 1998).

In the present study, we investigated whether the phosphorylation state of VASP might serve as a biochemical marker of the activity state of the vasoprotective NO/cGMP pathway in the intact vessel wall. The regulation of VASP phosphorylation in the intact rabbit vessel wall was analyzed using a monoclonal antibody specific for serine 239-phosphorylated VASP (Smolenski et al., 1998) as well as different inhibitors and an activator of the NO/cGMP signaling pathway. We also studied the distribution of VASP between vascular endothelium and vascular wall using a monoclonal antibody that recognizes an epitope present both in phospho- and dephospho-VASP (Abel et al., 1996).

Experimental Procedures

Materials. Anti-mouse immunoglobulin horse-radish peroxidase conjugate was purchased from DAKO (Hamburg, Germany), and Western blotting detection reagents from Amersham Biosciences (Freiburg, Germany). The monoclonal antibody 16C2, specific for P_{Ser239}-VASP, has been described previously (Smolenski et al., 1998); the monoclonal antibody IE273 against human VASP has also been reported (Abel et al., 1996). The monoclonal antibody directed against actin was purchased from Oncogene Research Products (Boston, MA). Phenylephrine (PE) and 1*H*-[1,2,4]oxadiazolo[3,4-*a*]quinoxalin-1-one (ODQ) were obtained from Hoechst Marion Roussel (Frankfurt, Germany); 8-bromo-cAMP (8-Br-cAMP) was obtained from Alexis (Lausen, Switzerland); and β-phenyl-1,N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer (*R*p-8-Br-PET-cGMPS) was obtained from BIOLOG (Bremen, Germany). All other chemicals were of the highest purity grade available and were obtained from either Sigma Chemicals (Deisenhofen, Germany) or Merck AG (Darmstadt, Germany). Water was deionized to 18 MΩ/cm (Milli-Q; Millipore, Eschborn, Germany). ODQ was dissolved in dimethyl sulfoxide (DMSO) and further diluted in Krebs-Henseleit buffer (KHB). The final DMSO concentration in superfusion experiments was 0.1% (v/v). In those experiments in which ODQ was present, controls also contained 0.1% (v/v) DMSO. DMSO itself sharply decreased the basal levels of P_{Ser239}-VASP to 27.7 ± 20% of control.

Isolated Blood Vessels and Incubations. After i.v. administration of heparin (500 units) and an overdose of sodium pentobarbitone (80 mg/kg), rabbits (1.8–2.3 kg) of either sex and mixed strains were sacrificed by exsanguination (Galle et al., 1995). The thoracic aorta was removed and placed in KHB (118.3 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 11.1 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 24 mM glucose, 26 μM EDTA pH 7), cleaned of fat and connective tissue, and cut into ring segments (3–4 mm), some of which were additionally denuded of the endothelium as indicated. In these cases, the endothelium was removed mechanically by inserting the tip of a pair of forceps into the lumen and rolling the tissue back and forth several times on a paper towel moistened with physiological salt solution. The endothelial integrity of the preparations or the absence of endothelium was determined by adding acetylcholine (1 μM) to the superfusate. Only arteries with a vasodilator response of > 70% inhibition of preconstriction were considered endothelium-intact (Galle et al., 1999). Each segment was mounted into thermostatically controlled (37°C) 2-ml tissue baths and connected to a strain gauge force transducer (Hugo Sachs, March-Hugstetten, Germany) to record changes in isometric tension. Each aortic ring was superfused at a constant rate (3 ml/min) with KHB (37°C) gassed with 5% CO_2/95% O_2 (Galle et al., 1995;1999). At the beginning of each experiment, rings were stretched to a resting tension of 2.5 g to optimize the following vasomotor responses. Subsequently, 1 μM phenylephrine (PE) was added for 30 min in the absence or presence of 1 mM N^{ω}-nitro-L-arginine methyl ester (L-NAME), 50 μM ODQ, or 30 μM *R*p-8-Br-PET-cGMPS. Dilator responses were induced by superfusion with 1 μM sodium nitroprusside (SNP) or 100 μM 8-Br-cAMP for 8 min.

Detection of VASP and P_{Ser239}-VASP by Immunoblotting. PE-contracted or fully relaxed rings were quickly removed from the tissue baths and homogenized in Laemmli buffer (Laemmli, 1970) preheated to 95°C. This method was validated against rapid freezing in liquid nitrogen followed by tissue homogenization in a liquid nitrogen steel mortar, yielding similar results (VASP and P_{Ser239}-VASP levels). Samples were then denatured in hot Laemmli buffer for 10 min, centrifuged, and the supernatant fractions (20 μg of protein per lane) separated by SDS-PAGE (Laemmli, 1970) using 12% gels. Proteins were electrophoretically transferred onto nitrocellulose membranes, blocked for 1 h at room temperature in Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Tween 20 and 3% fat-free dried milk, and then incubated

overnight at 4°C either with the 16C2 monoclonal antibody (1:2000) or the monoclonal antibody directed against actin (1:8000; Oncogene Research Products). The 16C2 antibody is directed against the phosphopeptide sequence RKVpS(239)KQE, which represents the VASP phosphorylation site at serine 239 (Smolenski et al., 1998). Alternatively, nitrocellulose blots were blocked for 1 h at room temperature in phosphate-buffered saline (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH 7.3) containing 1% bovine hemoglobin, 0.3% Triton X-100, and 0.05% Tween 20 and then incubated overnight at 4°C with the IE273 monoclonal antibody (1:2000) directed against the sequence ESVRRPWE of human VASP (Abel et al., 1996). Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. As a positive control for P_{Ser239}-VASP, 5 μg of protein from SNP-treated human platelets was used. In those aortic segments in which total VASP was detected (Fig. 2), 5 μg of protein from untreated human platelets was used.

Protein Quantification. Protein concentrations were determined by a modified Lowry method after protein precipitation with trichloroacetic acid (Peterson, 1977).

Signal Quantification. Proteins were visualized on photographic film using the ECL immunodetection kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Immunopositive enhanced chemiluminescence signals [absorbance over background] were quantified densitometrically using the NIH Image 1.59 software (http://rsb.info.nih.gov/nih-image/) (Sutherland et al., 1991; Correa-Rotter et al., 1992). The intensity of the P_{Ser239}-VASP and total VASP bands in the treated samples was expressed as a percentage of that of control samples.

Statistical Analysis. Data are presented as means ± S.E.M. Statistical differences between the means were analyzed by Student's unpaired *t* test (Figs. 1B, 2B, and 6B). For multiple comparisons, one-way analysis of variance (ANOVA) followed by Bonferroni's test was employed (Figs. 3C and 4B). Both analyses were carried out by using the Prism 2.0 software package (GraphPad Software, San Diego, CA). Probabilities of less than 5% ($p < 0.05$) were considered to be statistically significant.

Results

Vessel Wall P_{Ser239}-VASP Is Present Only in Aortic Rings with an Intact Endothelium. The 16C2 monoclonal antibody specific for P_{Ser239}-VASP only recognized a polypeptide of 46 kDa comigrating with a P_{Ser239}-VASP standard in homogenates of endothelium-intact, but not in endothelium-denuded rabbit aortic rings (Fig. 1A, upper bands). Precontraction with 1 μM PE had no effect on the phosphorylation of VASP at serine 239 (Fig. 1). The presence of basal P_{Ser239}-VASP levels only in those rabbit aortic segments that had an intact endothelium suggested either that VASP is primarily expressed in the vascular endothelium or that the phosphorylation of VASP at serine 239 in smooth muscle depends on the endothelium, or both.

Distribution of VASP between Vascular Endothelium and Vascular Wall. To examine whether VASP is primarily expressed in the vascular endothelium, we determined the amount of total VASP in crude homogenate of vascular wall with or without endothelium using the monoclonal antibody IE273 directed against VASP. Removal of the endothelium significantly ($p < 0.001$) decreased VASP levels to 35 ± 9% of control (Fig. 2B). Similarly, Oelze et al. (2000) recently reported a decrease of total VASP to 61 ± 6% of control after endothelial denudation. These differences may be attributable to the use of different endothelial denudation protocols or rabbit strains rather than different homogenization protocols, because we did not find significant differences in total VASP or P_{Ser239}-VASP in samples homogenized according to both protocols (data not shown). Nevertheless, these data consistently point toward relatively high VASP

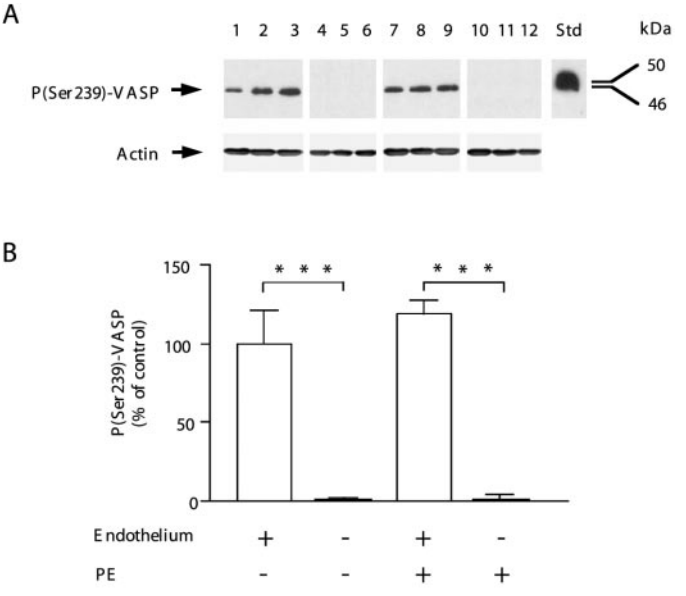

Fig. 1. Endothelium-dependent phosphorylation of VASP at serine 239 in rabbit aorta. Rabbit aortic rings with (lanes 1–3 and 7–9) or without (lanes 4–6 and 10–12) endothelium were incubated for 30 min at 37°C in the absence (lanes 1–6) or presence (lanes 7–12) of 1 μM PE. Segments were homogenized as described. To analyze VASP phosphorylation at serine 239, homogenates (20 μg of protein) were subjected to SDS-PAGE and immunoblotting using the monoclonal antibody 16C2. A, representative original blot for P_{Ser239}-VASP (upper bands) and actin (lower bands); B, corresponding densitometric quantification of P_{Ser239}-VASP. Similar levels of actin in all cases confirmed equal protein loading. Std, P_{Ser239}-VASP standard (5 μg of protein of homogenate of SNP-treated human platelets). Data shown in B are means ± S.E.M. of three experiments. Asterisks indicate statistically significant differences ($p < 0.001$; Student's unpaired *t* test).

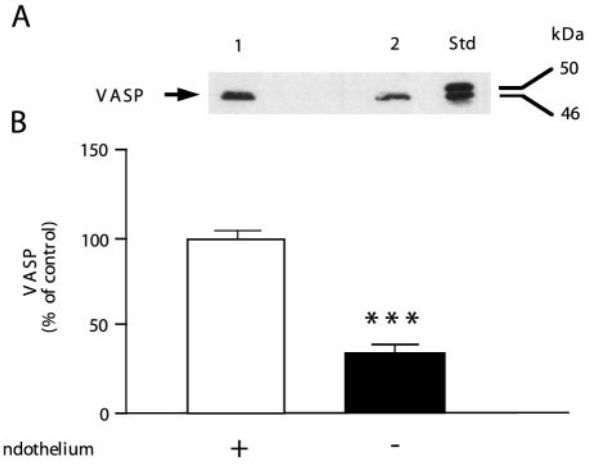

Fig. 2. Distribution of VASP in the rabbit aortic vascular wall. Intact rabbit aortic segments (lane 1) or segments denuded of endothelium (lane 2) were incubated for 30 min at 37°C with 1 μM PE. Segments were homogenized as described. To evaluate the content of total VASP, homogenates (20 μg of protein) were subjected to SDS-PAGE and immunoblotting using the monoclonal antibody IE273, which is specific for a phosphorylation-independent epitope of VASP. A, representative original blot; B, corresponding densitometric quantification. Std, VASP standard (5 μg of protein of homogenate of untreated human platelets). Data shown in B are the means ± S.E.M. of nine experiments. Asterisks indicate statistically significant difference ($p < 0.001$; Student's unpaired *t* test).

levels in the vascular endothelium. Phosphorylation of VASP at serine 157, as indicated by the presence of 50-kDa VASP (see standard from platelets in Fig. 2A), was not detectable in these samples.

SNP Increases the Levels of P_{Ser239}-VASP Even in the Absence of Endothelium. To investigate whether the phosphorylation of VASP at serine 239 in smooth muscle depends on the endothelial signaling pathway involving NO/cGMP, we analyzed the effects of the endothelium-independent sGC activator SNP on the formation of P_{Ser239}-VASP. SNP (1 μM), an NO donor, substantially enhanced the phosphorylation of VASP at serine 239 in endothelium-intact [177 ± 7% of control; Fig. 3, A, B (lane 2), and C] and endothelium-denuded aortic rings precontracted with PE [120 ± 21% of control; Fig. 3, B (lane 5) and C]. Because the level of VASP phosphorylation in denuded vessels was unexpectedly high (about two thirds of vascular VASP is removed by denudation), we hypothesized that the presence of endothelium would constitutively down-regulate sGC activity. However, when we used 1 μM SNP to relax endothelium-intact aortic rings that had been precontracted with PE and immediately thereafter denuded of endothelium, similar levels of P_{Ser239}-VASP [117 ± 35.6% of control; Fig. 3, B (lane 3) and C] were found compared with blood vessels first denuded and then exposed to SNP. Therefore, it can be excluded that the absence or presence of endothelium had any acute effects on vascular smooth muscle sGC.

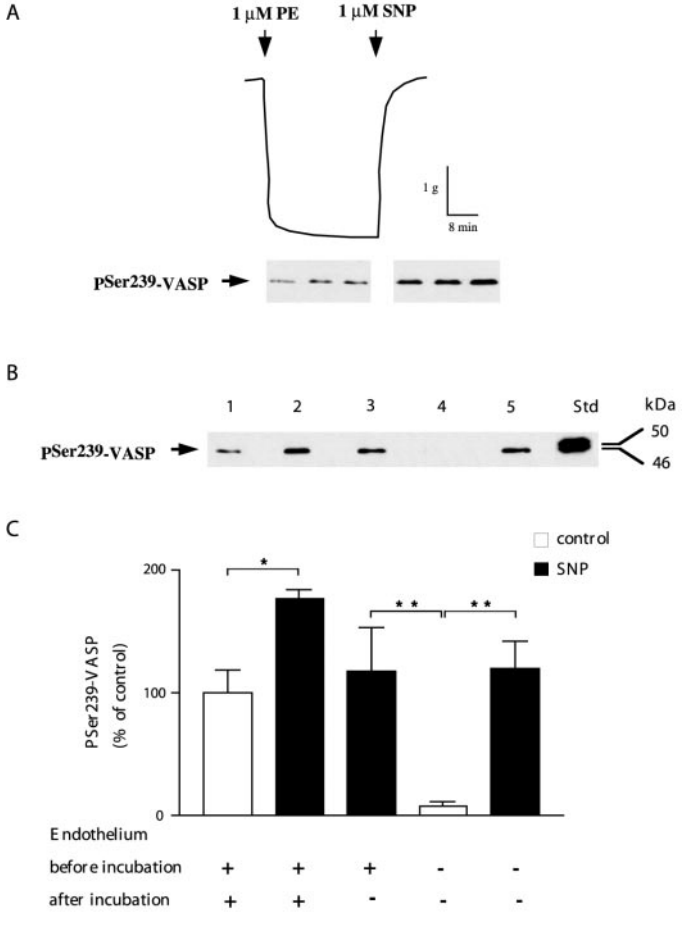

Fig. 3. SNP-induced phosphorylation of VASP at serine 239 in rabbit aorta with and without endothelium. A, representative original tracing of contraction with 1 μM PE for 30 min followed by relaxation with 1 μM SNP for 8 min of a rabbit endothelium-intact aortic ring showing the respective phosphorylation state of VASP at serine 239 according to Western blotting (bottom, $n = 3$). B and C, all aortic samples were incubated with 1 μM PE for 30 min at 37°C. Endothelium-intact (lane 1, $n = 6$; lane 2, $n = 15$) or endothelium-denuded (lane 4, $n = 6$; lane 5, $n = 15$) segments were incubated with (■) or without (□) 1 μM SNP for an additional 8 min. For one condition, the endothelium was removed after this additional SNP treatment (lane 3; $n = 9$). Segments were homogenized as described. To detect P_{Ser239}-VASP, homogenates (20 μg of protein) were subjected to SDS-PAGE and immunoblotting using the monoclonal antibody 16C2. B, representative original blot; C, corresponding densitometric quantification. Std, P_{Ser239}-VASP standard (5 μg of protein of homogenate of SNP-treated human platelets). Data in C represent means ± S.E.M. Asterisks indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$; Bonferroni's test).

8-Br-cAMP Enhances VASP Phosphorylation at Serine 239 but Not at Serine 157 in an *R*p-8-Br-PET-cGMPS Inhibitable Manner. In human platelets, the increase in cAMP levels induced by forskolin, an activator of adenylyl cyclase, and subsequent activation of cAK causes a shift in the apparent mobility of VASP from 46 to 50 kDa due to additional phosphorylation at serine 157 (Butt et al., 1994; Reinhard et al., 1999). Activation of cGK by cGMP after treatment with NO donors can also cause phosphorylation of VASP at this site, albeit to a lesser extent (Butt et al., 1994; Smolenski et al., 1998). However, the 50-kDa form of VASP was neither detected by the IE273 antibody (Fig. 2) nor induced by SNP (Fig. 3). Therefore, we wondered whether 8-Br-cAMP (100 μM) would be able to induce VASP phosphorylation at serine 157. We observed that 8-Br-cAMP, like SNP, increased only P_{Ser239}-VASP levels to 233 ± 38% of control in endothelium-intact aortic rings (Fig. 4). Because it was previously reported that cAMP might also cross-react with the cGK pathway (Lincoln et al., 1990; Jiang et al., 1992), we investigated whether the increased VASP phosphorylation levels at serine 239 induced by 8-Br-cAMP were caused by a direct activation of cAK or by a cross-reaction with cGK. Preincubation of aortic segments with the cGK inhibitor, *R*p-8-Br-PET-cGMPS inhibited significantly the formation of P_{Ser239}-VASP elicited by 8-Br-cAMP. Importantly, 8-Br-cAMP or 8-Br-cAMP/*R*p-8-Br-PET-cGMPS did not affect total VASP levels detected with antibody IE273 (Fig. 4A, bottom).

Dephosphorylation Kinetics. P_{Ser239}-VASP, formed in response to SNP treatment, was dephosphorylated in homogenates from endothelium-intact and denuded rabbit aorta when incubation of aortic rings with SNP was followed by an intensive washout (Fig. 5). VASP phosphorylation levels stayed almost unchanged during the first 4 min of washout, but rapidly declined during the ensuing washout phase. Thus, phosphorylation of VASP at serine 239, both in vascular endothelium and the entire vascular wall, depends on endothelial factors that can be mimicked by exogenous NO. Also, phosphorylation is fully reversible when NO is eliminated.

Inhibition of the NO/cGMP Pathway by L-NAME or ODQ Substantially Decreases the Content of P_{Ser239}-VASP in the Intact Aortic Vessel Wall. Previous studies using cGK-I-deficient cells established that NO/cGMP-induced VASP phosphorylation is primarily mediated by cGK-I (Draijer et al., 1995; Massberg et al., 1999), and it has additionally been demonstrated that the absence of cGK-I leads to a complete disruption of the NO/cGMP signaling pathway in aortic rings (Pfeifer et al., 1998). Therefore, we investigated whether inhibition of the NO/cGMP pathway contrib-

utes in a similar way to a decrease in VASP phosphorylation at serine 239 in endothelium-intact rabbit aorta. VASP phosphorylation was analyzed in aortic segments incubated in the absence or presence of the NOS inhibitor L-NAME (1 mM) or the sGC inhibitor ODQ (50 μM). L-NAME and ODQ dramatically reduced P_{Ser239}-VASP levels to 20.7 ± 6.1 and 15.6 ± 4.1% of control, respectively (Fig. 6). Neither inhibitor altered total VASP levels as detected with the antibody IE273 (Fig. 6A, bottom). Thus, phosphorylation of VASP at serine 239 is regulated primarily by NO in intact vascular tissue.

Discussion

The NO/cGMP and PGI_2/cAMP pathways regulate vascular smooth muscle tone and proliferation (Furchgott and Vanhoutte, 1989). Dysfunction of these pathways is implicated in a variety of vascular disease states (Somlyo and Somlyo, 1994; Kojda and Harrison, 1999). Considerable evidence supports the view that the effects of cGMP within the vessel wall are mediated by cGK-I (Pfeifer et al., 1998; Hofmann et al., 2000). To further analyze the physiological and pathophysiological mechanisms at work within the vascular wall, it is important to identify biochemical markers that provide direct information about the activation state of these pathways. Measurement of cGMP and cAMP levels has been employed to assess the activities of the NO/cGMP and PGI_2/cAMP pathways (Rasmussen et al., 1990; Murad, 1994). Recently, phosphorylation of VASP at serine 239 (P_{Ser239}-VASP) was also proposed to be a sensitive monitor of defective NO/cGMP signaling and endothelial dysfunction (Oelze et al., 2000). In this study, we further investigate P_{Ser239}-VASP as an alternative biochemical marker for the integrity of the NO/cGMP pathway.

Basal P_{Ser239}-VASP levels are detectable only in endothelium-intact rabbit aorta (Fig. 1). This is consistent with NOS-III being constitutively active in vascular endothelium and continuously producing NO, which stimulates sGC and subsequently leads to the production of cGMP. In turn, this second messenger activates cGK, which phosphorylates VASP at serine 239.

The presence of VASP in both vascular smooth muscle and vascular endothelium is in agreement with recent studies showing VASP expression in rat, mouse, and human vascular smooth muscle (Markert et al., 1996; Mönks et al., 1998; Aszódi et al., 1999; Hauser et al., 1999) as well as vascular endothelial cells (Draijer et al., 1995; Markert et al., 1996; Mönks et al., 1998; Hauser et al., 1999). In our experiments, however, the endothelium seemed to contain an unexpectedly high amount of VASP (about 65%) relative to the total vessel wall, which is in agreement with results reported previously (about 44%) (Oelze et al., 2000). Differences are probably

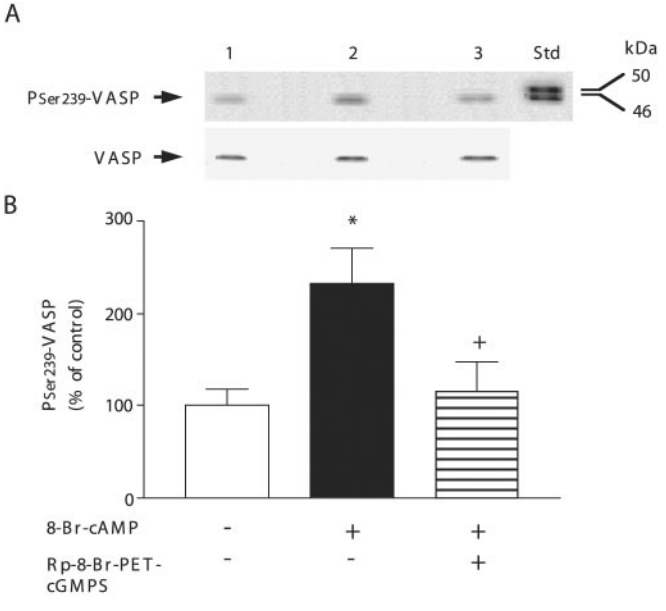

Fig. 4. Enhancement of P_{Ser239}-VASP formation in the presence of 8-Br-cAMP and inhibition of this phosphorylation by *R*p-8-Br-PET-cGMPS. Endothelium-intact rabbit aortic segments were incubated at 37°C for 30 min with 1 μM PE (lane 1; $n = 6$) or with 1 μM PE plus 30 μM *R*p-8-Br-PET-cGMPS and thereafter additionally with 100 μM 8-Br-cAMP (lanes 2 and 3, respectively; $n = 6$) for 8 min. Segments were homogenized as described. To analyze phosphorylation of VASP at serine 239, homogenates (20 μg of protein) were subjected to SDS-PAGE and immunoblotting. Representative original blots using the monoclonal antibodies 16C2 (A, top) and IE273 (A, bottom). B, corresponding densitometric quantification of A, top. Std, P_{Ser239}-VASP standard (5 μg of protein of homogenate of SNP-treated human platelets). Data represent means ± S.E.M. Asterisk and cross indicate statistically significant differences [*, $p < 0.05$ versus control (□) and +, $p < 0.05$ versus 8-Br-cAMP treated aortic rings (■); Bonferroni's test]. Note that 8-Br-cAMP treatment did not generate a VASP band comigrating with the 50-kDa form of platelet VASP indicative of serine 157 phosphorylation.

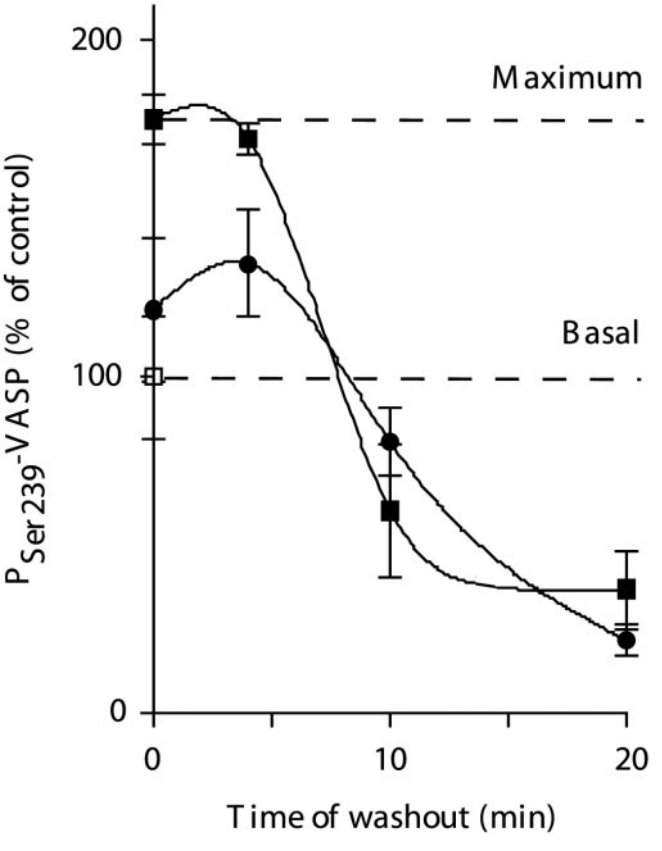

Fig. 5. Dephosphorylation of P_{Ser239}-VASP in endothelium-intact and -denuded rabbit aorta after SNP washout. Endothelium-intact (■) or -denuded (●) aortic rings were precontracted with 1 μM PE for 30 min at 37°C and then relaxed with 1 μM SNP for 8 min. SNP was then eliminated from the medium followed by an intensive washout (0, 4, 10 and 20 min; ■, $n = 3$ and ●, $n = 6$). At the end of washout, segments were homogenized as described previously. To detect P_{Ser239}-VASP, homogenates (20 μg of protein) were subjected to SDS-PAGE and immunoblotting using the monoclonal antibody 16C2. □, the basal VASP phosphorylation at serine 239 in endothelium-intact rabbit aorta ($n = 9$).

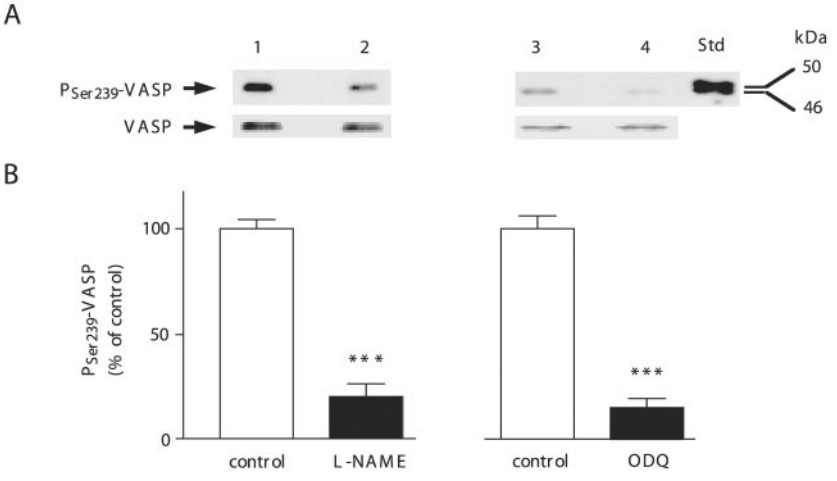

Fig. 6. NO-dependent phosphorylation of vessel-wall VASP at serine 239. Endothelium-intact rabbit aortic rings were incubated for 30 min at 37°C with 1 μM PE alone (lane 1; $n = 12$) or together with 1 mM L-NAME (lane 2; $n = 12$) or 50 μM ODQ (lane 4; $n = 6$). DMSO [0.1% (v/v)], which was the solvent for ODQ, was present also in control samples (lane 3, $n = 6$). All segments were homogenized as described. To measure VASP phosphorylation at serine 239, homogenates (20 μg of protein) were subjected to SDS-PAGE and immunoblotting. Representative original blots using the monoclonal antibodies 16C2 (A, top) and IE273 (A, bottom). B, corresponding densitometric quantification of A, top. Std, P_{Ser239}-VASP standard (5 μg of protein of homogenate of SNP-treated human platelets). Data represent means ± S.E.M. Asterisks indicate statistically significant differences [$p < 0.001$ versus control (□); Student's unpaired t test].

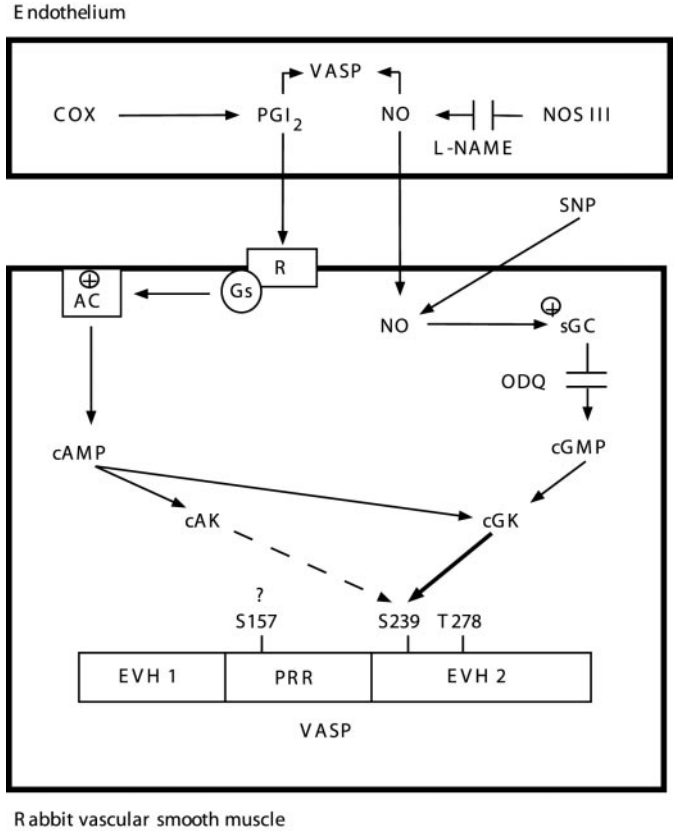

Fig. 7. Pathways involved in the regulation of VASP phosphorylation at serine 239 in rabbit aorta. Vascular smooth muscle VASP is primarily phosphorylated at serine 239 by cGK after activation of the NO/cGMP pathway. In this study with rabbit aorta, a stimulus- or drug-induced appearance of the 50-kDa form of VASP, usually indicative of serine 157 phosphorylation, was never observed. This suggests that VASP serine 157 phosphorylation was either not present or not detectable under the conditions tested (see text). COX, cyclooxygenase; EVH, Ena-VASP homology domain; Gs, stimulatory G-protein; PRR, proline-rich region.

caused by the different endothelial denudation protocols or rabbit strains employed. The significant increase in the level of P_{Ser239}-VASP by SNP, even in the absence of endothelium, suggests that the dramatic decrease in P_{Ser239}-VASP levels in endothelium-denuded blood vessels was caused, in part, by the lack of endothelial factors, which could be replaced by exogenous NO donors such as SNP. Interestingly, the response of endothelium-denuded aortic segments to SNP produced twice as much P_{Ser239}-VASP as expected from its VASP content. As a possible explanation for this, vascular smooth muscle VASP may be phosphorylated with higher efficiency than endothelial VASP in blood vessels, because smooth muscle cells contain significantly higher amounts of cGK than endothelial cells (Oelze et al., 2000). Moreover, very little is known about the affinities of cGK for different substrates in intact cells and their regulation.

The increased phosphorylation of VASP at serine 239 elicited by SNP was fully reversed after 10 min of washout either in endothelium-intact or -denuded rabbit aorta. This showed that, in endothelium-intact rabbit aorta, SNP induced P_{Ser239}-VASP levels decreased more slowly after SNP washout than cGMP levels, which reach basal levels after 5 min (Galle et al., 1999). These results suggest that, as is the case with platelets, blood vessels dynamically phosphorylate/dephosphorylate VASP after a stimulus.

In vitro and in intact cells of many species, cAK/cGK phosphorylation of VASP induces a marked shift in its apparent molecular mass from 46 to 50 kDa when analyzed by SDS-PAGE (Reinhard et al., 1999). This electrophoretic shift of VASP is caused by additional phosphorylation at serine 157 (Butt et al., 1994; Smolenski et al., 1998). However, in our experiments with rabbit intact blood vessels, 8-Br-cAMP (Fig. 4A) or SNP (Fig. 3B) induced the phosphorylation of VASP at serine 239 but not at serine 157, as observed in platelets. Using purified catalytic subunit of cAK and homogenates from rabbit aorta, we also failed to detect the presence of phosphorylated VASP at serine 157 by Western blotting using the IE273 antibody (data not shown). Moreover, the fact that we detected only the 46-kDa form of VASP is in agreement with other reports in which only this form of VASP in rabbit aortic (Oelze et al., 2000) and rat neointimal tissue (Mönks et al., 1998) was found. There are several plausible explanations for this observation. The amino acid sequences of human, canine, and mouse VASP, but not that of rabbit, are known. It is therefore possible that the serine 157 phosphorylation site is not present in rabbit VASP (Fig. 7). Alternatively, if this residue is present, the serine 157-induced shift of rabbit VASP in SDS-PAGE may be too small to be resolved from serine 157-dephospho-VASP. It is unlikely that phosphorylation of this site is prevented by other factors present in the rabbit vascular wall, because human

platelet VASP can efficiently be phosphorylated at serine 157 by cAK even in rabbit aortic homogenates spiked with traces of human platelet homogenate (data not shown). The highly conserved serine 239 phosphorylation site of VASP is present and phosphorylated by both cAMP- and cGMP-dependent pathways in the rabbit vessel wall. The possibility that 8-Br-cAMP induced VASP phosphorylation at serine 157 but not at serine 239 was ruled out, because under our conditions, the anti-VASP antibody IE273 detected only the 46-kDa and not the 50-kDa form of VASP phosphorylated at serine 157 (Figs. 2A, 4A, and 6A). Inhibition of 8-Br-cAMP–induced P_{Ser239}-VASP increase caused by *R*p-8-Br-PET-cGMPS suggest that cAMP cross-reacts with the cGK pathway (Lincoln et al., 1990; Jiang et al., 1992), increasing cGK-mediated phosphorylation of VASP at serine 239. It is unlikely that under these conditions cAK might be also inhibited, because in human platelets, 100 μM *R*p-8-Br-PET-cGMPS inhibited only cGK activation, without affecting activation of cAK (Butt et al., 1995). However, we cannot, of course, fully exclude a direct activation of the PGI_2/cAMP/cAK pathway contributing to VASP phosphorylation at this site.

A previous study with cGK-I–deficient mice showed a complete disruption of the NO/cGMP signaling pathway in vascular smooth muscle, which indicated that effects of cGMP are primarily mediated by cGK-I (Pfeifer et al., 1998). Consistent with this study, we found that inhibition of the NO/cGMP pathway with either L-NAME (20.7 ± 6.1% of control) or ODQ (15.6 ± 4.1% of control) markedly reduced basal P_{Ser239}-VASP levels. Our results are in agreement with a previous report that 3 mM N^{ω}-nitro-L-arginine reduced P_{Ser239}-VASP to about 35% of control (Oelze et al., 2000) and suggest that VASP is primarily phosphorylated at serine 239 by the NO/cGMP/cGK pathway in the intact vessel wall.

Importantly, when analyzed with the antibody IE273, similar amounts of VASP were detected in the homogenates from treated compared with untreated endothelium-intact aortic rings (Figs. 4 and 6), indicating that the compounds tested (L-NAME, ODQ, 8-Br-cAMP, and *R*p-8-Br-PET-cGMPS) affected only VASP phosphorylation but not total VASP levels.

The functional role of VASP in the regulation of the vascular vessel wall remains to be elucidated (Aszódi et al., 1999; Hauser et al., 1999). Recent evidence suggests that vascular smooth muscle contractility is independent of VASP, because aortic rings derived either from wild type or VASP-deficient mice contracted with similar sensitivity and effectiveness when treated with agonists and relaxed identically when treated with cGMP or cAMP analogs (Aszódi et al., 1999). However, Mena and/or Evl (which are beyond detection limits in mouse platelets) may compensate for the loss of VASP in the vascular wall. This hypothesis is further supported by the finding that human VASP can rescue the lethal phenotype of *D. melanogaster* Ena-null mutants (Ahern-Djamali et al., 1998).

In conclusion, our findings suggest that VASP is phosphorylated by the NO/cGMP pathway primarily at serine 239, which is reversible and can be dephosphorylated by removing NO. P_{Ser239}-VASP can thus serve as a biochemical marker for the activity state of the NO/cGMP pathway in endothelial function in vivo.

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