

Atrial Natriuretic Peptide-initiated cGMP Pathways Regulate Vasodilator-stimulated Phosphoprotein Phosphorylation and Angiogenesis in Vascular Endothelium*

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Nitric oxide (NO)- and atrial natriuretic peptide (ANP)-initiated cGMP signaling cascades are important in the maintenance of cardiovascular homeostasis. The molecular signaling mechanisms downstream of cGMP are not well understood, however. We have used small interfering RNA (siRNA) approaches to specifically knock down a series of signaling proteins in bovine aortic endothelial cells, and we have combined biochemical analyses with physiological assays to investigate cGMP-mediated signal transduction pathways. Activation of particulate guanylate cyclase (GC-A) by ANP leads to a substantial, dose-dependent, rapid, and sustained increase in intracellular cGMP. In contrast, stimulation of soluble guanylate cyclase by NO yields only a weak and transient increase in cGMP. ANP-induced cGMP production is selectively suppressed by siRNA-mediated knockdown of GC-A. ANP greatly enhances the phosphorylation at Ser-239 of the vasodilator-stimulated phosphoprotein (VASP), a major substrate of cGMP-dependent protein kinase (PKG) that significantly influences actin dynamics. Moreover, the ANP-induced phosphorylation of VASP at Ser-239 is accompanied by increased actin stress fiber formation and enhanced endothelial tube formation. siRNA-mediated knockdown of GC-A, VASP, or PKG abolishes ANP-induced VASP Ser-239 phosphorylation, stress fiber formation, and endothelial tube formation. We have demonstrated similar findings in human umbilical vein endothelial cells, where ANP substantially enhances intracellular cGMP content, phosphorylation of VASP at Ser-239, and endothelial tube formation. Taken together, our findings suggest that ANP-mediated cGMP signal transduction pathways regulate PKG phosphorylation of VASP Ser-239 in endothelial cells, resulting in reorganization of the actin cytoskeleton and enhancement of angiogenesis.

cGMP is involved in numerous vascular signaling pathways, and abnormalities in these pathways have been implicated in the pathophysiology of hypertension, atherosclerosis, and diabetes (1, 2). In endothelial cells, cGMP is synthesized by two distinct guanylate cyclase (GC)⁵ isoforms, the nitric oxide (NO)-activated soluble GC (sGC) and the atrial natriuretic peptide (ANP)-activated particulate GC (GC-A) (2–4). NO, produced by endothelial NO synthase (eNOS), plays a crucial role in a variety of vascular responses such as blood pressure control, platelet aggregation, and vascular smooth muscle cell proliferation (5). Activation of eNOS is strongly influenced by a diverse collection of cell surface receptors, including vascular endothelial growth factor (VEGF) receptor (KDR) and sphingosine 1-phosphate (S1P) receptor S1P₁ (Edg-1) (6, 7). ANP is released primarily by cardiac atria in response to atrial stretch; this peptide binds with high affinity to the membrane receptor guanylate cyclase GC-A located in renal cells and in the vasculature (8, 9). Activated GC-A promotes diuresis and inhibits the renin-angiotensin-aldosterone system, thereby controlling salt and water retention and blood pressure (10). Recent studies have revealed ANP to be an endogenous vasoprotective substance as well as a potent pharmacological agent in the treatment of cardiovascular diseases (10–12). GC-A is abundantly expressed in the vascular endothelium, and binding of ANP to GC-A significantly increases intracellular cGMP levels (13–15).

The principal effectors of cGMP in the cardiovascular system include cGMP-gated monovalent cation channels, phosphodiesterases, and cGMP-dependent protein kinases (PKGs) (16, 17). Two mammalian PKG genes have been identified, encoding PKG I and PKG II. PKG I is the prevalent PKG isoform expressed in the cardiovascular system (17) and will be referred to here as PKG. Although the functions of PKG have been probed both with PKG knock-out mice and with analyses of cellular substrates phosphorylated by PKG, much remains to be understood about the molecular and cellular significance of PKG signaling in endothelial homeostasis. PKG knock-out mice display decreased life span, impaired vascular smooth

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⁵ The abbreviations used are: GC, guanylate cyclase; ANP, atrial natriuretic peptide; VASP, vasodilator-stimulated phosphoprotein; NO, nitric oxide; siRNA, small interfering RNA; sGC, soluble guanylate cyclase; PKG, cGMP-dependent protein kinase; eNOS, endothelial NO synthase; BAEC, bovine aortic endothelial cells; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; S1P, sphingosine 1-phosphate; SNP, sodium nitroprusside; GFR, growth factor-reduced; FOV, fields of view; PKA, cAMP-dependent protein kinase; ERK, extracellular signal-regulated kinase.

muscle cell relaxation, diminished ischemia-induced angiogenesis, and disturbed platelet activation, highlighting the importance of this enzyme in the cardiovascular system (18, 19). However, analyses of PKG substrate proteins have suggested multiple, and sometimes contradictory, roles of PKG in vascular cell signaling (19).

One prominent substrate of PKG is the vasodilator-stimulated phosphoprotein (VASP), a member of the family of actin-binding regulatory proteins termed Ena/VASP. In vascular endothelial cells, overexpression of VASP has been shown to induce stress fiber formation and membrane ruffling (20). VASP activity is regulated through phosphorylation by cAMP-dependent protein kinases (PKA) and PKG at distinct sites (21–24). Phosphorylation of VASP may affect its capacity to associate with actin filaments and antagonize filament branching (25, 26). Although the mechanism of VASP regulation of the cytoskeleton is still controversial, it is widely accepted that VASP activation has multiple effects on the physiological processes governed by cellular actin networks, such as cell motility, migration, angiogenesis, and vascular permeability (27–30). VASP-deficient mice show an augmented early vascular injury response, potentially because of enhanced platelet adhesion, but appear to be protected from progression of long term renal disease by enhanced preservation and regeneration of renal endothelial capillaries (21, 31). Reduced phosphorylation of VASP may provide a link between reduced cGMP signaling and vascular dysfunction in model systems of late stage atherosclerosis (1). Multiple studies have identified the level of phosphorylation of VASP as a sensitive monitor of endothelial dysfunction and vascular oxidative stress (32, 33).

Although reports have established that both NO and ANP initiate cGMP signaling in endothelial cells, the relative contributions of each in cGMP-mediated pathways are not completely understood (14, 34, 35). Furthermore, studies of angiogenesis have addressed the role of NO- but not ANP-induced cGMP signaling. This study uses duplex small interfering RNA (siRNA) targeting constructs to specifically knock down a series of key signaling proteins in bovine aortic endothelial cells (BAEC) and to explore the resulting changes in receptor-mediated cGMP and downstream signaling pathways. In both BAEC and human umbilical vein endothelial cells (HUVEC), we observe that ANP-activated GC-A generates a strong and sustained cGMP response, whereas NO-activated sGC elicits only a weak and transient cGMP signal. siRNA-mediated knockdown of GC-A selectively suppresses the ANP-induced cGMP response, and sodium nitroprusside (SNP) enhancement of intracellular NO content has little effect on the cGMP response. ANP greatly enhances the phosphorylation of VASP at Ser-239 and augments endothelial tube formation and actin stress fiber formation. siRNA-mediated knockdown of GC-A, PKG, or VASP differentially modulates endothelial tube formation. Moreover, knockdown of GC-A or PKG abolishes VASP Ser-239 phosphorylation as well as ANP-induced endothelial tube formation and actin stress fiber formation. Our results suggest that ANP-mediated cGMP signal transduction in the vascular endothelium is an important regulator of cytoskeletal organization and angiogenesis.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Lipofectamine™ 2000 and most cell culture reagents were from Invitrogen. Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT). M199 medium and endothelial cell growth supplement were from Sigma. Polyclonal antibodies against phospho-VASP (Ser-239), phospho-VASP (Ser-157), VASP, phospho-Akt (Ser-473), Akt, phospho-eNOS (Ser-1179), and phospho-ERK1/2 (Thr-202/Thr-204) were from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal antibodies against eNOS and VASP were from BD Transduction Laboratories. The SuperSignal chemiluminescence detection reagents and secondary antibodies conjugated with horseradish peroxidase were from Pierce. Cyclic GMP enzyme immunoassay kit was from Cayman Chemical (Ann Arbor, MI). Protein concentrations were determined using protein assay kits from Bio-Rad. VEGF and wortmannin were from Calbiochem. S1P was from Biomol (Plymouth Meeting, PA). ANP (rat) and all other reagents were from Sigma.

Culture and Treatment of Cells—BAEC were from Cell Applications (San Diego) and were maintained as described (36). HUVEC were from Genlantis, Inc. (San Diego), and were maintained in M199 medium supplemented with 50 mg/ml endothelial cell growth supplement and 20% FBS. BAEC between passages 5 and 8, or HUVEC between passages 3 and 4, were incubated in serum-free media overnight prior to all experiments. Treatment with ANP, VEGF, or S1P and subsequent cell lysate preparation were performed as described previously (36, 37), with corresponding vehicle treatments as controls.

siRNA Preparation and Transfection—Consistent with previous work (38, 39), we designed a GC-A siRNA duplex corresponding to bases 1767–1785 from the open reading frame of the bovine GC-A mRNA (GenBank™ accession number XM_612318), 5'-CGAACACUUGACCAGGUUU-dTdT-3'; a sGC siRNA duplex corresponding to bases 77–95 from the open reading frame of the bovine sGC mRNA (GenBank™ accession number Y_00770), 5'-AAAAAGAGGCACAAU-AGA-dTdT-3'; a PKG I(α/β) siRNA duplex corresponding to bases 979–997 from the open reading frame of the bovine PKG mRNA (GenBank™ accession number NM_174436), 5'-GGAUUAGAUGAUGUUUCUA-dTdT-3'; and a VASP siRNA duplex corresponding to bases 965–982 from the open reading frame of the bovine VASP mRNA (GenBank™ accession number NM_001038110), 5'-GGAUGAAGUCG-UCUUCUUC-dTdT-3'. The siRNA duplex oligonucleotides were from Ambion, Inc. (Austin, TX). The nonspecific control siRNA was from Dharmacon, Inc. (Lafayette, CO), 5'-AUUGUAUGCGAUCGCAGAC-dTdT-3'. BAEC were transfected with siRNA as described (37).

Cyclic Nucleotide and Western Blot Analyses—cGMP level was determined using the cGMP enzyme immunoassay kit (Cayman Chemical) according to the manufacturer's protocol. Cells were treated with isobutylmethylxanthine (200 μ M) for 10 min prior to agonist stimulation. Protein concentration was determined according to the Bradford method, using parallel wells of cells. Western blot analyses were performed as

described previously (37). Statistical analyses for biochemical assays were performed using one-way analysis of variance, followed by Tukey's test for multiple comparisons. A p value of <0.05 was considered significant. All experiments were repeated at least three times.

Endothelial Tube Formation Assay—250 μ l of Matrigel or growth factor-reduced (GFR) Matrigel (BD Biosciences) was deposited into each well of a 24-well plate and allowed to coagulate for 30 min at 37 °C. For studies of BAEC without ANP treatment, cells were resuspended in 10% FBS-supplemented media, and 5×10^4 cells were added to each Matrigel-coated well. For studies of BAEC with ANP treatment, cells were resuspended in serum-free media, and 3×10^4 cells were added to each GFR Matrigel-coated well in the presence or absence of 10 nM ANP. For studies of HUVEC with ANP treatment, cells were resuspended in HUVEC culture medium with 5% FBS and added to Matrigel-coated wells in the presence or absence of 10 nM ANP. These cells were then incubated on Matrigel for 9–12 h at 37 °C and imaged by phase contrast microscopy (Nikon Eclipse TS100, 5x objective). Six random fields of view (FOV) per well were examined. For quantification purposes, a node was defined as an aggregation of cells from which three or more tube-like structures originated, and a tube referred to a continuous stretch of at least two cells containing no more than two nodes. For each FOV, ImageJ (National Institutes of Health) was used to measure the total tube length and the length per tube in units of pixels. The number of tubes per FOV was also counted. Each experiment was repeated in 9–12 wells. Data were pooled and analyzed using one-way analysis of variance, followed by Student's t test for paired comparisons. A p value of < 0.05 was considered significant. To prevent the variability among different lots of Matrigel or GFR Matrigel from affecting the results, each series of experiments used Matrigel from the same lot.

Laser Confocal Microscopy of the Actin Cytoskeleton—BAEC grown on coverslips were transfected with control or target protein-specific siRNA using Lipofectamine 2000. 48 h after transfection, cells were treated, fixed, stained, and optically analyzed by laser confocal microscopy, as described previously (37).

RESULTS

Comparison of ANP- and NO-stimulated cGMP Responses—cGMP is generated through the activation of both ANP/GC-A and NO/sGC, and the relative contribution of each pathway was measured in BAEC after stimulation by ANP, VEGF, or S1P. ANP elicited an immediate and substantial increase in intracellular cGMP content, reaching a maximum within 2 min and declining slightly to a steady high level over the next 60 min (Fig. 1A). To identify the contributions of the two distinct GC isoforms in this response, we selectively knocked down the expression of GC-A or sGC in BAEC by transfecting a custom-designed siRNA duplex targeted to the bovine GC-A mRNA or the bovine sGC mRNA, respectively. Consistent with the known activation of GC-A by ANP, the ANP-stimulated cGMP response was suppressed by knockdown of GC-A but not by knockdown of sGC (Fig. 1B). Knockdown of VASP had no significant effect on the ANP-stimulated cGMP response (data

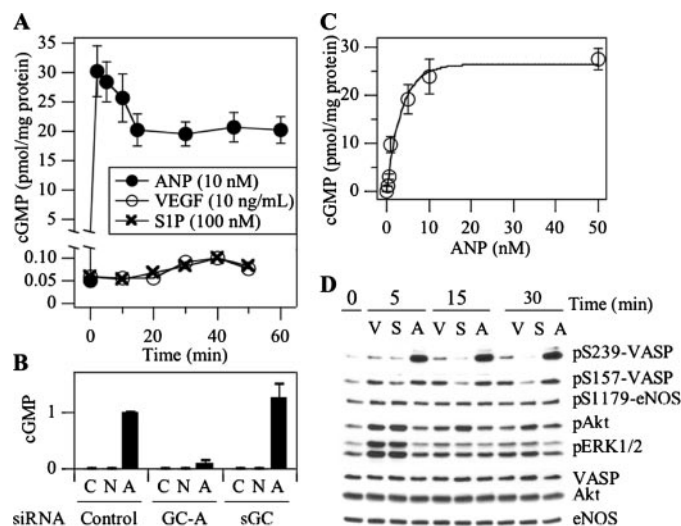


FIGURE 1. Differential cGMP responses to ANP and NO in endothelial cells. A, intracellular cGMP content was determined in BAEC at the indicated times of treatment with 10 nM ANP (solid circles), 10 ng/ml VEGF (open circles), or 100 nM S1P (crosses). B, intracellular cGMP content was determined in BAEC transfected with control, GC-A, or sGC siRNA, and treated with vehicle (C), 1 μ M SNP (N), or 10 nM ANP (A). For comparison, cGMP measurements in pmol/mg protein were normalized to the cGMP level (16.2 ± 4.1 pmol/mg protein) in BAEC transfected with control siRNA and treated with 10 nM ANP (control siRNA, A). C, cGMP response to different concentrations of ANP was assessed in BAEC. Cells were treated with ANP for 20 min prior to measurements. Error bars represent S.E. derived from three independent experiments. D, BAEC were treated with 10 ng/ml VEGF (V), 100 nM S1P (S), or 1 nM ANP (A), and the quantity of phospho-VASP at Ser-239 (pS239-VASP), phospho-VASP at Ser-157 (pS157-VASP), phospho-eNOS at Ser-1179 (pS1179-eNOS), phospho-Akt at Ser-473 (pAkt), phospho-ERK1/2 at Thr-202/Tyr-204 (pERK1/2), VASP, Akt, and eNOS was determined at the indicated times using immunoblotting.

not shown). In contrast to the rapid and robust ANP-induced response, VEGF- or S1P-stimulated NO production generated only a delayed and very weak cGMP response (Fig. 1A). Furthermore, treatment of cells with the direct NO donor sodium nitroprusside (SNP) had little effect on intracellular cGMP levels (Fig. 1B). Analysis of a concentration-response curve revealed that cGMP production reached half-maximum at an EC_{50} of 2.3 ± 0.4 nM ANP ($n = 3$) (Fig. 1C).

We next investigated the phosphorylation of various proteins downstream of cGMP to characterize the roles of ANP/GC-A and NO/sGC in cGMP signaling. Phosphorylation of VASP at Ser-239 (pS239-VASP) was greatly enhanced by ANP but only modestly enhanced by VEGF or S1P (Fig. 1D). VASP Ser-239 is known to be preferentially phosphorylated by PKG, whereas Ser-157 is preferentially phosphorylated by PKA (24, 25). We assessed cAMP levels in BAEC at different time points up to 30 min after treatment with 10 nM ANP. We found that ANP had little effect on cAMP levels (data not shown), and ANP-induced VASP phosphorylation at Ser-157 was much less than that at Ser-239 (Fig. 1D). Together, these findings indicate that ANP-induced signaling is primarily modulated through PKG. We also examined the effects of ANP, VEGF, and S1P on other key endothelial signaling proteins, and we used phospho-specific antibodies to probe immunoblots of agonist-treated endothelial cells. We analyzed phosphorylation of eNOS at Ser-1179 and protein kinase Akt at Ser-473, two key targets of the phosphatidylinositol 3-kinase pathway, and phosphorylation of the extracellular signal-regulated kinases (ERK) 1/2, which

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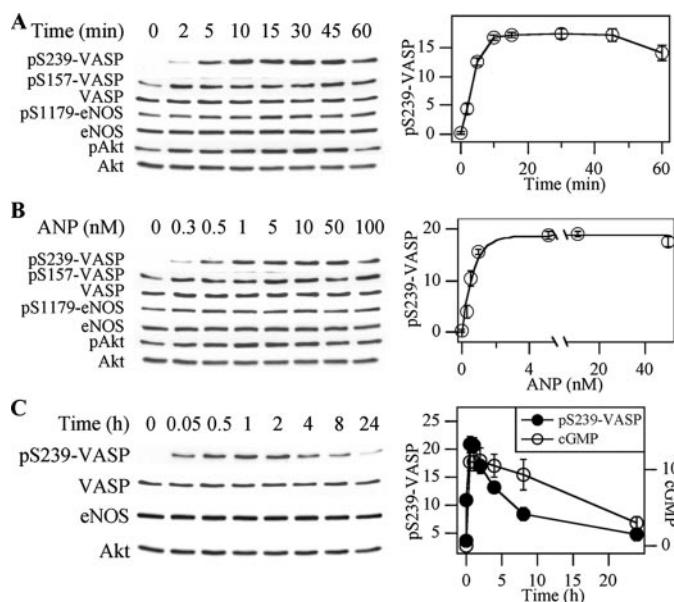


FIGURE 2. ANP-induced phosphorylation responses and sustained cGMP signaling in endothelial cells. ANP-treated BAEC were analyzed in immunoblots probed with antibodies directed against pS239-VASP, pS157-VASP, pS1179-eNOS, or pAkt. Equal loading was confirmed by immunoblotting with anti-VASP, anti-eNOS, and anti-Akt antibodies. Shown on the left are representative blots and on the right are quantitative plots derived from pooled data. Each point in the graphs represents the mean \pm S.E. of three independent experiments. *A*, BAEC were treated with 1 nM ANP for the indicated times. Right panel shows densitometry of pS239-VASP staining as a function of treatment time. *B*, BAEC were treated with the indicated concentrations of ANP for 20 min. Right panel shows densitometry of pS239-VASP staining as a function of treatment time. *C*, BAEC were treated with 10 nM ANP for the indicated times up to 24 h. Right panel shows densitometry of pS239-VASP staining on the left axis (solid circles) and the corresponding cGMP content on the right axis (open circles).

reflects activity of the mitogen-activated protein kinase (MAPK) pathway. Fig. 1D shows a small increase in eNOS phosphorylation in response to ANP, VEGF, and S1P. In multiple experiments, and found that the ANP effect on eNOS phosphorylation was subtle, variable, and statistically insignificant, whereas we and others (37, 40–43) have extensively characterized a significant eNOS phosphorylation response to VEGF and S1P. Additionally, Fig. 1D shows significant phosphorylation of Akt and ERK1/2 in response to VEGF and S1P. In all cases, ANP induced little or no change in the phosphorylation of these important constituents of the nitric oxide signaling pathway. Immunoblot analyses established that the abundance of VASP, Akt, and eNOS proteins remained constant during these treatments (Fig. 1D).

ANP Modulates Phosphorylation of VASP at Ser-239—We next measured the time course and concentration-response of VASP phosphorylation in ANP-stimulated BAEC. ANP-induced enhancement in pS239-VASP reached a maximum at 10 min and then remained at this level for up to an hour (Fig. 2A). The time course of VASP Ser-239 phosphorylation was similar to that of ANP-stimulated cGMP production, with a several-minute delay. The EC_{50} for VASP Ser-239 phosphorylation, $\sim 0.4 \pm 0.1$ nM ANP, was also similar to that for cGMP production (Fig. 2B). The ANP-induced phosphorylation of VASP at Ser-157 (pS157-VASP), a preferred site for phosphorylation by PKA, was much less marked than that at the PKG site Ser-239

(Fig. 2). In addition, the phosphorylation of eNOS at Ser-1179 and of Akt was only nominally affected by ANP, suggesting that ANP plays a minimal role in the activation of eNOS (Fig. 2). The total protein expression of VASP, eNOS, and Akt remained constant in these experiments.

ANP Induces a Long Term cGMP Signaling Response—Short term time courses showed that ANP-modulated responses were maintained at an enhanced level for up to 1 h in BAEC, suggesting a persistent effect. Therefore, we probed ANP-induced cGMP signaling for longer time periods to determine the duration of these responses. We found that the ANP-induced elevation in pS239-VASP persisted for up to 8 h and required up to 24 h to return to basal levels (Fig. 2C). We also examined the accumulation of cGMP in BAEC after extended exposure to ANP, and we found that cGMP content displayed a temporal response similar to that of pS239-VASP. Shortly after the addition of ANP, cGMP content climbed sharply to a peak value. The increase in cGMP accumulation was maintained for up to 8 h and declined back to basal levels by 24 h (Fig. 2C).

Targeted Knockdown of GC-A or PKG by Duplex siRNA Constructs Abrogates ANP-induced Enhancement of cGMP Responses in BAEC—We selectively knocked down the expression of GC-A in BAEC using the siRNA duplex targeted to the bovine GC-A mRNA. Although the efficiency of GC-A knockdown could not be assessed because of the lack of suitable antibodies, immunoblot analyses showed that VASP Ser-239 phosphorylation was completely abolished in cells transfected with GC-A siRNA (Fig. 3A). We next designed and transfected an siRNA duplex targeted to the bovine PKG I mRNA (Fig. 3, B and C), and found that PKG expression was suppressed in transfected cells, whereas the expression of total VASP remained constant (Fig. 3, B and C). pS239-VASP was completely absent in PKG siRNA-transfected BAEC, and no increase in VASP Ser-239 phosphorylation was observed in response to ANP (Fig. 3B), even after prolonged treatment at high ANP concentrations (Fig. 3C). We used a nonspecific siRNA as a negative control, and found that the phosphorylation of VASP in cells transfected with control siRNA was similar to that in nontransfected cells, thus verifying that the process of siRNA transfection did not significantly perturb VASP phosphorylation. Unlike the phosphorylation of VASP at Ser-239, the phosphorylation of VASP at Ser-157 was only partially affected by siRNA-mediated knockdown of GC-A or PKG. In these experiments, the expression of total VASP was unaffected by siRNA-mediated knockdown of GC-A or PKG (Fig. 3, A–C).

Knockdown of GC-A, PKG, or VASP Differentially Affects Endothelial Tube Formation in BAEC—We designed an siRNA duplex targeted to the bovine VASP mRNA. Transfection of VASP siRNA resulted in more than 90% knockdown of VASP expression in BAEC, whereas the expression of eNOS and β -actin was unaffected (Fig. 3D). We then used knockdown of GC-A, PKG, and VASP to explore the physiological correlates of ANP-regulated cGMP signaling in studies of angiogenesis, a complex multistep process that results in the formation of three-dimensional endothelial tubular structures (44–46). One of the most specific and widely applied assays of angiogenesis is the measurement of endothelial tube formation (47), which occurs spontaneously *in vitro* in the presence of appro-

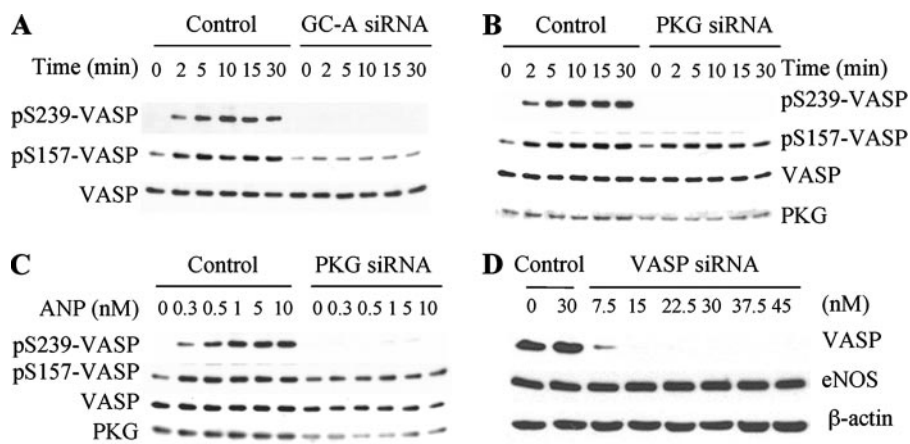


FIGURE 3. Effects of siRNA-mediated down-regulation of GC-A, PKG, and VASP. Shown are immunoblotting results representative of three independent experiments. A–C, siRNA-transfected BAEC were analyzed in immunoblots probed with antibodies directed against pS239-VASP or pS157-VASP. Immunoblotting with the anti-VASP antibody confirmed specificity of siRNA-mediated down-regulation. For all experiments involving siRNA, parallel experiments were performed under the same conditions using control siRNA-transfected cells. BAEC were transfected with control siRNA (A–C, left), GC-A siRNA (A, right), or PKG siRNA (B and C, right) and treated with 1 nM ANP for the indicated times (A and B) or with the indicated concentrations of ANP for 20 min (C). D, siRNA-mediated down-regulation of VASP was analyzed. BAEC were transfected with control (left) or VASP (right) siRNA at the indicated concentrations and analyzed in immunoblots probed with anti-VASP antibody. Immunoblotting with antibodies directed against eNOS and β -actin confirmed specificity of VASP siRNA.

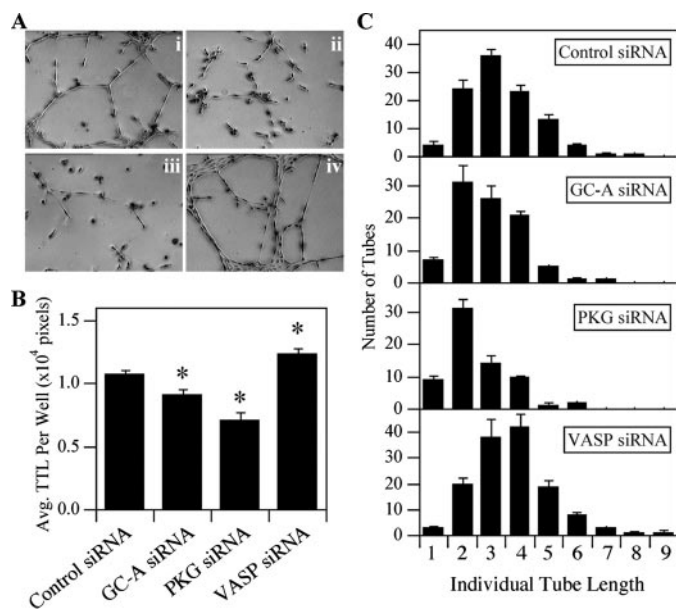


FIGURE 4. Endothelial tube formation following siRNA-mediated down-regulation of cGMP signaling proteins. BAEC were grown on Matrigel in 24-well plates and transfected with control, GC-A, PKG, or VASP siRNA. A, shown are representative images of endothelial tube formation by BAEC transfected with control siRNA (panel i), GC-A siRNA (panel ii), PKG siRNA (panel iii), and VASP siRNA (panel iv). B, average total tube length (TTL) per well is shown with S.E. derived from 12 independent experiments. Asterisk indicates statistically significant difference ($p < 0.05$) compared with the total tube length per well of BAEC transfected with control siRNA. C, individual tube length (in number of pixels) is grouped into nine categories: 1 (0–49), 2 (50–99), 3 (100–149), 4 (150–199), 5 (200–249), 6 (250–299), 7 (300–349), 8 (350–399), and 9 (400–449).

appropriate extracellular matrix components. Here we use the term “tube formation” to refer to the two-dimensional tubular network formed by endothelial cells on MatrigelTM. This is sometimes also referred to as “cord formation” (48, 49). We cultured BAEC on MatrigelTM matrix (50–52) and analyzed the effect of

down-regulation of ANP-modulated cGMP signaling on endothelial tube formation. Endothelial cells transfected with control siRNA formed a network of tube-like structures within 9–12 h of culture on Matrigel (Fig. 4A, panel i). siRNA-mediated knockdown of GC-A hindered tube formation (Fig. 4A, panel ii), and siRNA-mediated knockdown of PKG more severely impeded tube formation (Fig. 4A, panel iii). In contrast to the effect of GC-A and PKG knockdown, siRNA-mediated knockdown of VASP increased endothelial tube formation (Fig. 4A, panel iv).

We performed quantitative analyses to more comprehensively assess the structures formed by populations of siRNA-treated endothelial cells on Matrigel. For the purpose of these analyses, we defined a node to be an aggregation of endothelial cells from which three or more tube-like extensions originated, and a tube to be a continuous stretch of at least two endothelial cells containing no more than two nodes. Fig. 4B shows for each treatment the average values of total tube length per well, measured in units of pixels. Length per tube was also evaluated for each treatment, and the results are shown as histograms in Fig. 4C. We found that knockdown of GC-A or PKG reduced the total tube length (Fig. 4B) as well as the average length per tube (Fig. 4C). Conversely, knockdown of VASP enhanced the total tube length (Fig. 4B) as well as the average length per tube (Fig. 4C).

ANP-induced cGMP Signaling Modulates Endothelial Tube Formation—We next assessed the effect of ANP on endothelial tube formation. For these experiments, we plated BAEC onto growth factor-reduced Matrigel, which reduces basal tube formation by nontreated cells and thus accentuates agonist responses. We found that ANP promoted tube formation in BAEC transfected with control siRNA (Fig. 5A). Quantitative analysis showed that ANP enhanced both the total tube length (Fig. 5B) and the average length per tube (Fig. 5C). To explore the mechanism of the ANP-modulated angiogenic response, we evaluated the role of various proteins along the cGMP signaling pathway in endothelial tube formation. In cells treated with siRNA constructs targeting GC-A, PKG, or VASP, the ANP-induced enhancement in endothelial tube formation was abolished (Fig. 5B).

ANP Induces cGMP Signaling and Enhances Tube Formation in HUVEC—To explore whether our findings were specific to bovine endothelial cells, we assessed ANP-initiated signaling in HUVEC. Consistent with our findings in BAEC, treatment with VEGF or S1P induced only a relatively small increase in intracellular cGMP content, whereas treatment with ANP induced an immediate, sustained, and much larger increase in intracellular cGMP levels (Fig. 6A). Furthermore, the ANP-induced

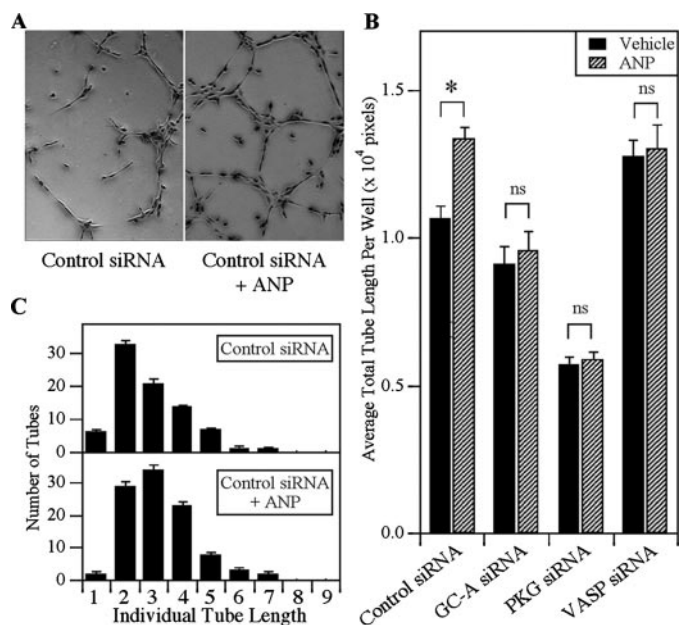


FIGURE 5. ANP-stimulated endothelial tube formation following siRNA-mediated down-regulation of cGMP signaling proteins. BAEC were grown on growth factor-reduced Matrigel in 24-well plates and transfected with control, GC-A, PKG, or VASP siRNA. *A*, shown are representative images of endothelial tube formation by nontreated (*left*) and 10 nM ANP-treated (*right*) BAEC transfected with control siRNA. *B*, average total tube length per well of nontreated (*solid bar*) and ANP-treated (*patterned bar*) cells is shown with S.E. derived from nine independent experiments. Asterisk indicates statistically significant difference ($p < 0.05$) compared with nontreated control siRNA-transfected cells. *ns* indicates that the value is not statistically significant compared with nontreated siRNA-transfected cells in each case. *C*, individual tube length (in number of pixels) is grouped into nine categories: 1 (0–49), 2 (50–99), 3 (100–149), 4 (150–199), 5 (200–249), 6 (250–299), 7 (300–349), 8 (350–399), and 9 (400–449).

cGMP response in HUVEC was more than 10-fold greater than that induced by the NO donor SNP (Fig. 6*B*). As we found for BAEC, ANP promoted a striking increase in phosphorylation of VASP at Ser-239, with only a modest enhancement of VASP phosphorylation by VEGF or S1P (Fig. 6*C*). Experiments using Matrigel demonstrated that ANP promoted tube formation in HUVEC (Fig. 6*D*, panels *i* and *ii*), similar to results obtained in BAEC (Fig. 5). Quantitative analysis showed that ANP enhanced both the total tube length (Fig. 6*D*) and the average length per tube (data not shown), as was also seen for BAEC (Fig. 5).

ANP-induced cGMP Signaling Modulates Actin Stress Fiber Formation—We next studied the effect of ANP on actin cytoskeleton reorganization in BAEC. Cells were fixed before or after treatment with ANP, and polymerized F-actin was labeled using fluorescently tagged phalloidin. Laser confocal microscopy images showed that ANP promotes actin stress fiber formation in nontransfected BAEC (Fig. 7, *A* and *E*). In cells transfected with siRNA constructs targeting GC-A, PKG, or VASP, the ANP-induced enhancement in actin stress fiber formation was abolished (Fig. 7).

DISCUSSION

cGMP signaling pathways are critically involved in the regulation of vascular homeostasis. Vascular smooth muscle cells are clearly a principal target for NO/cGMP signaling (53, 54), but cGMP signaling in endothelial cells is also emerging as a key

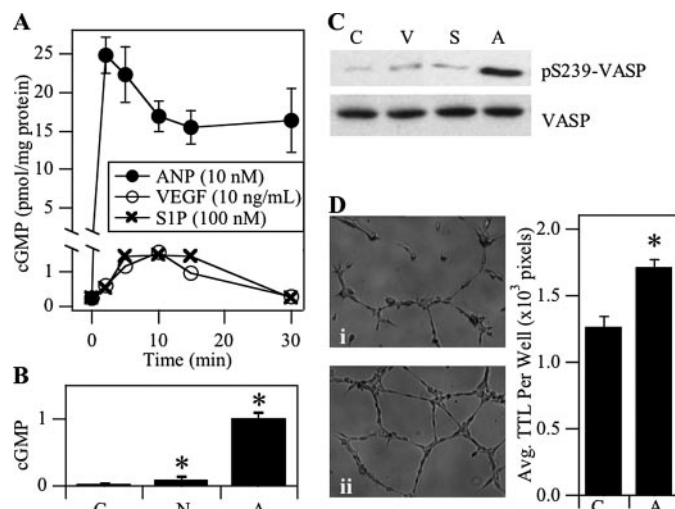


FIGURE 6. ANP-initiated cGMP signaling and tube formation in HUVEC. *A*, intracellular cGMP content was determined in HUVEC at the indicated times of treatment with 10 nM ANP (*solid circles*), 10 ng/ml VEGF (*open circles*), or 100 nM S1P (*crosses*). *B*, intracellular cGMP content was determined in HUVEC treated with vehicle (*C*), 1 μ M SNP (*N*), or 10 nM ANP (*A*). Asterisk indicates statistically significant difference ($p < 0.015$) compared with vehicle-treated cells. *C*, HUVEC were treated with vehicle (*C*), 10 ng/ml VEGF (*V*), 100 nM S1P (*S*), or 10 nM ANP (*A*) for 5 min, and the amounts of phospho-VASP at Ser-239 (*pS239-VASP*) and VASP were determined using immunoblotting. *D*, HUVEC were grown on Matrigel in 24-well plates. Shown on the *left* are representative images of endothelial tube formation by nontreated (*panel i*) and 10 nM ANP-treated (*panel ii*) HUVEC. Shown on the *right* is the average total tube length (TTL) per well of nontreated (*C*) and ANP-treated (*A*) cells, with S.E. derived from five independent experiments. Asterisk indicates statistically significant difference ($p < 0.02$) compared with nontreated cells.

regulator of vascular biology. Importantly, our studies reveal distinct roles for ANP/GC-A and NO/sGC activation in cGMP signaling in both intact bovine and human endothelial cells; the effects of ANP/GC-A pathway stimulation are significantly greater than those of NO/sGC pathway stimulation on both cGMP accumulation and PKG-mediated VASP phosphorylation at Ser-239 (Figs. 1 and 6). ANP binds to its membrane receptor GC-A, which catalyzes the conversion of GTP to cGMP. Accumulation of cellular cGMP activates downstream effectors including PKG, which selectively binds and phosphorylates various substrates (2, 55, 56). One major substrate of PKG is VASP, and the phosphorylation state of VASP is an effective predictor of endothelial function in model systems of atherosclerosis, hypertension, and nitrate tolerance (2, 29, 30). The physiological relevance of VASP phosphorylation provides an important link to our evaluation of the phosphorylation of VASP in BAEC as an indicator for the activity of PKG and cGMP signal transduction.

VASP has three phosphorylation sites, including the PKG preferential site Ser-239 and the PKA preferential site Ser-157. Our measurements show that ANP induction of VASP phosphorylation is much more prominent at Ser-239 than at Ser-157, suggesting that ANP-initiated signal transduction in BAEC propagates mainly through cGMP/PKG. ANP treatment of vascular endothelial cells prompts an immediate and robust accumulation of cGMP, and this response is further reflected in the rapid and dose-dependent increase in VASP phosphorylation at Ser-239 (Fig. 2). Furthermore, the ANP-induced

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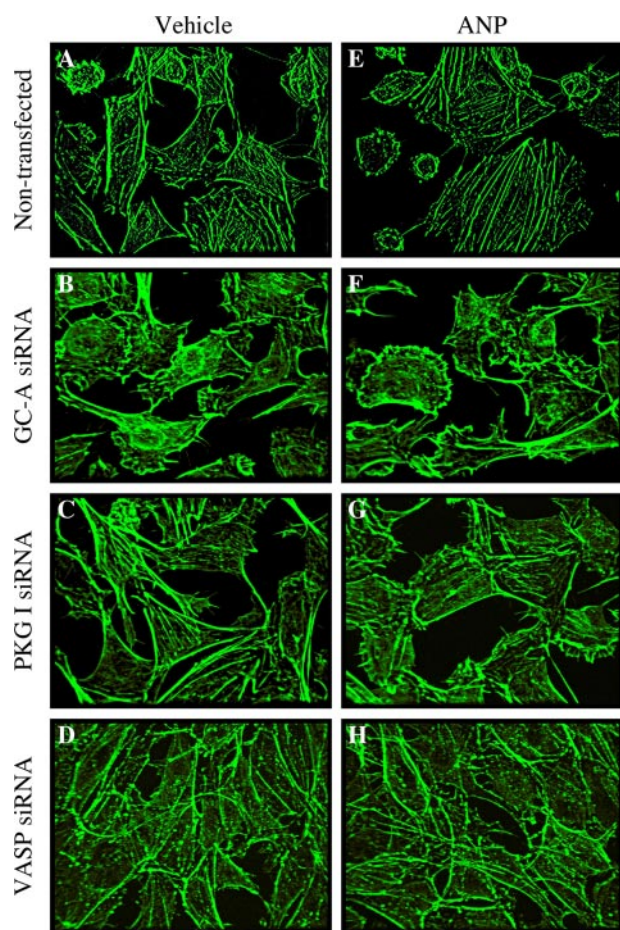


FIGURE 7. ANP-mediated regulation of actin stress fiber bundling in BAEC. BAEC were transfected with GC-A siRNA (B and F), PKG siRNA (C and G), or VASP siRNA (D and H). Nontreated (A–D) and ANP-treated (E–H) cells were fixed and labeled with Alexa 568-phalloidin. Shown are representative images of the actin cytoskeleton, in two-dimensional projections of three-dimensional optical stacks, obtained by laser confocal imaging and processed by deconvolution.

enhancement of pS239-VASP is sustained for a surprisingly long time, maintaining its intensity for at least 8 h (Fig. 2C). This prolonged phosphorylation of VASP at Ser-239 may be regulated in part by phosphodiesterases, because phosphodiesterases have been implicated in the temporal responses of endothelial cells to ANP stimulation (57).

We have applied siRNA-based technology to establish the roles of GC-A, PKG, and VASP in the ANP-initiated cGMP signaling pathway in vascular endothelial cells. siRNA-mediated knockdown of GC-A in BAEC completely eradicates the substantial ANP-induced increase in pS239-VASP observed in control cells (Fig. 3A). A similarly striking abrogation of ANP-induced pS239-VASP is observed in cells transfected with PKG siRNA (Fig. 3, B and C). The complete suppression of VASP phosphorylation at Ser-239 by siRNA-mediated knockdown of GC-A or PKG indicates that GC-A and PKG are crucial components of the ANP-initiated cGMP signaling pathway.

Previous studies of cGMP signal transduction have concentrated primarily on vascular smooth muscle cells (58, 59); the physiological consequences of cGMP signaling in endothelial cells are only now beginning to be more fully characterized. In the regulation of angiogenesis, the role of NO-initiated cGMP

signaling has been extensively explored (60–62), but the role of ANP-initiated cGMP signaling is less well understood (63, 64). We present here *in vitro* studies of endothelial tube formation in the physiologically relevant environment provided by Matrigel. Based on existing approaches (44, 45, 61), we have developed a quantitative analysis that can be reliably and universally applied to different cell culture samples. In addition to designing siRNA duplex constructs targeting GC-A and PKG, we have also constructed VASP siRNA, transfection of which dramatically reduces VASP expression levels without affecting the expression of eNOS and β -actin (Fig. 3D). We find that knockdown of GC-A or PKG reduces endothelial tube length, thus demonstrating the importance of cGMP signaling in tube formation (Fig. 4). ANP significantly enhances tube formation in both bovine and human endothelial cells, and this increase is because of an increase in the average length per tube rather than the number of tubes formed (Figs. 5 and 6). The latter finding suggests that ANP may promote endothelial proliferation at the tip of an angiogenic sprout, with no substantive change in endothelial branching (65). A previous study of fixed human microvascular endothelial cells grown on GFR Matrigel found that ANP had little effect on tube formation (66). However, significant differences in experimental conditions, both for cell culture and for the analysis of tube formation, make it difficult to compare those results with our present findings, in which we show an important effect of ANP on endothelial tube formation. siRNA-mediated knockdown of GC-A, PKG, or VASP prevents the ANP-induced increase in total tube length (Fig. 5B), implying that activation of the ANP-initiated cGMP signaling pathway is crucial to ANP modulation of endothelial tube formation. Interestingly, cells transfected with VASP siRNA display augmented total tube length. Together with the observed ANP induction of tube formation, this result suggests that endothelial tube formation by BAEC may be negatively modulated by VASP, and that PKG-induced phosphorylation of VASP may enhance angiogenesis. Mounting evidence suggests that angiogenesis is regulated by cytoskeletal alterations that lead to changes in cell morphology and in the rate of cell migration and proliferation (45, 67–69). Because VASP has the ability to bind cytoskeletal proteins and regulate actin cytoskeleton dynamics (24, 25), we have explored the role of ANP-induced VASP phosphorylation in cGMP-modulated endothelial tube formation. Previous studies have reported conflicting observations (27, 63, 64, 70), suggesting that ANP-induced VASP phosphorylation may have complicated consequences. Our results show that activation of cGMP signaling induces both VASP phosphorylation at Ser-239 and actin stress fiber formation (Fig. 7E). Moreover, siRNA-mediated knockdown of GC-A, PKG, or VASP prevents the increase in stress fiber formation, suggesting a crucial role for ANP-initiated cGMP signaling in the modulation of the actin cytoskeleton (Fig. 7). The regulation of actin organization in cells is a complex and dynamic process involving many parameters. Our studies have revealed intricate relationships among ANP-initiated signaling, actin dynamics, and endothelial tube formation.

Taken together, the present studies show that ANP stimulates the production of cGMP and activates the cGMP signaling cascade, leading to the phosphorylation of VASP by PKG in

cultured endothelial cells. In turn, VASP modulates actin cytoskeleton reorganization (20, 26, 29, 30, 56) and perturbs the physiological behavior of endothelial cells. We show that ANP preferentially promotes the phosphorylation of VASP at Ser-239 and that ANP enhances actin stress fiber formation and endothelial tube formation. siRNA-mediated suppression of cGMP signaling abrogates these ANP-induced effects. These studies provide novel insights into the mechanisms underlying the differential roles of receptor-modulated cGMP signaling pathways in endothelial cells.

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