Atrial Natriuretic Peptide–Mediated Inhibition of Microcirculatory Endothelial Ca\textsuperscript{2+} and Permeability Response to Histamine Involves cGMP-Dependent Protein Kinase I and TRPC6 Channels


Objective—Histamine increases microvascular endothelial leakage by activation of complex calcium-dependent and -independent signaling pathways. Atrial natriuretic peptide (ANP) via its cGMP-forming guanylyl cyclase-A (GC-A) receptor counteracts this response. Here, we characterized the molecular mechanisms underlying this interaction, especially the role of cGMP-dependent protein kinase I (cGKI).

Approach and Results—We combined intravital microscopy studies of the mouse cremaster microcirculation with experiments in cultured microvascular human dermal endothelial cells. In wild-type mice, ANP had no direct effect on the extravasation of fluorescent dextran from postcapillary venules, but strongly reduced the histamine-provoked vascular leakage. This anti-inflammatory effect of ANP was abolished in mice with endothelial-restricted inactivation of GC-A or cGKI. Histamine-induced increases in endothelial [Ca\textsuperscript{2+}] were markedly attenuated by the Ca\textsuperscript{2+}-entry inhibitor SKF96365 and in mice with ablated transient receptor potential canonical (TRPC) 6 channels. Conversely, direct activation of TRPC6 with hyperforin replicated the hyperpermeability responses to histamine. ANP, via cGKI, stimulated the inhibitory phosphorylation of TRPC6 at position Thr\textsuperscript{60} and prevented the hyperpermeability responses to hyperforin. Moreover, inhibition of cGMP degradation by the phosphodiesterase 5 inhibitor sildenafil prevented the edematic actions of histamine in wild types but not in mice with endothelial GC-A or cGKI deletion.

Conclusions—ANP attenuates the inflammatory actions of histamine via endothelial GC-A/cGMP/cGKI signaling and inhibitory phosphorylation of TRPC6 channels. The therapeutic potential of this novel regulatory pathway is indicated by the observation that sildenafil improves systemic endothelial barrier functions by enhancing the endothelial effects of endogenous ANP. (Arterioscler Thromb Vase Biol. 2013;33:2121-2129.)

Key Words: atrial natriuretic factor ■ cyclic GMP ■ cyclic GMP dependent protein kinase I ■ endothelial cells ■ guanylyl cyclase A ■ TRPC6 channel

Atrial natriuretic peptide (ANP), via its cGMP-forming guanylyl cyclase-A (GC-A) receptor, has a critical role in the regulation of arterial blood pressure and blood volume.\textsuperscript{1} The GC-A receptor is densely expressed on vascular endothelia, but the biological function is controversial.\textsuperscript{2} To elucidate whether the endothelial effects of ANP participate in the regulation of systemic blood pressure and volume, we generated mice with conditional, endothelial cell (EC)-restricted ablation of the GC-A gene (EC GC-A KO mice).\textsuperscript{3} Comparative intravital microscopy studies in these and control mice together with MRI demonstrated that ANP, via GC-A, mildly stimulates systemic transendothelial albumin transport in the microvasculature of skeletal muscle and skin.\textsuperscript{4,5} Together with many other studies, our observations support the notion that concerted renal diuretic/natriuretic and mild systemic endothelial hyperpermeability actions of ANP are essential to adjust intravascular fluid volume.\textsuperscript{6}

However, this notion apparently contradicts published in vitro and in vivo studies showing that ANP can attenuate pulmonary endothelial hyperpermeability induced by inflammatory...
mediators, such as lipopolysaccharide or tumor necrosis factor α, or by hypoxia (reviewed in Kuhn). Mechanistic studies indicated that ANP prevents activation of Rho-A and Rho-kinase (ROCK) signaling and abrogates ROCK-induced activation of myosin phosphatase (MYPT1), phosphorylation of myosin light chain, and pulmonary endothelial contraction. These seemingly different findings (systemic intravascular volume—regulating hyperpermeability actions versus pulmonary barrier–protecting effects) suggested that ANP either acts differently on systemic versus lung endothelial barrier functions or it exerts dual, opposite effects on quiescent endothelia (mild hyperpermeability) and an inflammation-activated endothelium (barrier stabilization).

Increased endothelial permeability is also characteristic of many systemic diseases, including allergic responses, atherosclerosis, tumor growth, edema, and sepsis. In particular, histamine strongly increases systemic endothelial permeability within minutes. Activation of endothelial Gq-coupled H1 receptors activates phospholipase Cβ and elevates intracellular [Ca2+]. Independently from this canonical pathway, histamine stimulates RhoA/ROCK-mediated inhibition of MYPT1. Ultimately, both pathways increase myosin light chain kinase activity and trigger actin-myosin contraction, thereby increasing paracellular permeability.

Here, we combined studies in cultured microvascular endothelial cells and imaging of vascular permeability in the mouse cremaster microcirculation of various monogenic mouse models to investigate the hypothesis that ANP counteracts not only pulmonary but also systemic inflammation, that is, histamine-induced hyperpermeability. In particular, we generated a new mouse model with conditional, endothelial-specific inactivation of cGMP-dependent protein kinase I (cGKI), to test the role and targets of this kinase in the mediation of the endothelial effects of ANP. Our findings reveal that transient receptor potential canonical (TRPC) 6 channels are essential for the hyperpermeability effects of histamine. Most importantly, they identify a regulatory pathway by which histamine-induced activation of TRPC6 channels and subsequent calcium-dependent acute endothelial hyperpermeability are prevented by ANP/GC-A–induced, cGKI-mediated inhibitory phosphorylation of these channels. Finally, our data show that the drug sildenafil improves microvascular endothelial barrier functions primarily by enhancing the endothelial actions of endogenous ANP.

Materials and Methods
Materials and Methods are provided in the online-only Supplement.

Results
ANP Prevents the Activation of Perivascular Mast Cells and Mast Cell–Induced Vascular Leakage

As a measure of microvascular permeability, leakage of fluorescein isothiocyanate (FITC)-dextran from postcapillary venules to the interstitial space of the cremaster muscle was analyzed by intravital fluorescence microscopy in anesthetized mice. Baseline FITC-dextran extravasation was very low and was not affected by ANP (100 nmol/L; Figure 1A). To study whether ANP can modulate an acute inflammatory response, first, we superfused the m. cremaster with the mast cell secretagogue compound 48/80 (5 μg/mL for 10 minutes). Mast cell degranulation was assessed by adding ruthenium red to the superfusate 10 minutes later. As shown in Figure 1, C48/80 provoked a very strong, sudden, and reversible extravasation of FITC-dextran (Figure 1A), together with intense mast cell degranulation (Figure 1B). Notably, ANP markedly prevented the stimulatory effects of C48/80, both on mast cell degranulation (Figure 1A) and on microvascular permeability (Figure 1A and 1B).

Next, we tested whether ANP interferes with the hyperpermeability effects of histamine, as a main mediator released by mast cells. Indeed, ANP pretreatment (20 minutes) significantly attenuated the acute hyperpermeability effects of histamine (local superfusion with 2 μmol/L histamine during 10 minutes, in the presence of ANP; Figure 2A). Notably, this antihistaminergic effect of ANP was abolished in mice with endothelial-restricted deletion of the ANP-receptor, GC-A (EC GC-A KO mice); Figure 2B). We conclude that ANP has no direct effect on baseline FITC-dextran leakage; however, the peptide, via its endothelial GC-A receptor and cGMP formation, prevents histamine-induced acute vascular hyperpermeability.
The Antihistaminergic Actions of ANP Are Mediated by Endothelial cGKI

Several cGMP-modulated proteins are expressed in endothelial cells, such as phosphodiesterases (PDE 2 and 3, which modulate cAMP levels) and cGMP-dependent protein kinase I (cGKI; reviewed in Reference 2). To study the role of cGKI, here, we generated a new genetic mouse model with endothelial-restricted inactivation of this kinase. Mice with floxed cGKI gene were mated with Tie2-Cre transgenic mice. This cross generated mice with endothelial-restricted inactivation of the cGKI gene. Of the F1 generation, 45% of the mice were assigned to the Tie2-Cre+/-cGKIfl/fl genotype, whereas expression levels were almost fully abolished in MLECs from EC cGKI KOs (Figure 3A). We used intravital microscopy to directly observe the cremaster microcirculation. In control mice, histamine-induced strong leakiness of FITC-dextran from postcapillary venules, and ANP reduced this effect (Figure 3C). In EC cGKI KO mice, the inflammatory action of histamine was unaltered; however, ANP did not prevent the hyperpermeability actions of histamine (Figure 3D). These results indicate that activation of cGKI mediates the acute anti-inflammatory actions of the endothelial ANP/GC-A/cGMP signaling pathway.

ANP, Via cGMP/cGKI Signaling, Diminishes the Ca2+ Responses to Histamine

The following experiments in cultured human dermal microvascular (HDM) ECs aimed to dissect the distal molecular pathway(s) mediating the antihistaminergic effects of ANP/cGKI. As shown in Figure 4A, ANP (0.1–1000 nmol/L) for 10 minutes, in the presence of the nonselective PDE inhibitor IBMX) increased the cGMP content of HDMECs, demonstrating the expression of the GC-A receptor. Western blot analyses showed cGKI expression and activity (demonstrated

Figure 2. Atrial natriuretic peptide (ANP), via the endothelial guanylyl cyclase-A (GC-A) receptor, prevents histamine-induced acute vascular leakage of fluorescein isothiocyanate (FITC)-dextran. Time course of changes in permeability during 20 minutes of continuous local ANP (100 nmol/L) superfusion in control mouse (GC-A+/+; A) and in mice with conditional, endothelial GC-A deletion (EC GC-A KO; B). n=7–8 mice per genotype and treatment; *P<0.05 vs vehicle. Top in A and B, Representative original photographs.

Figure 3. The antihistaminergic actions of atrial natriuretic peptide (ANP) are mediated by endothelial cGMP–dependent protein kinase I (cGKI). A, cGKI was detected in microvascular lung endothelial cells (MLECs) from control mice (cGKI+/+) but was almost absent in cultured MLEC preparations from EC cGKI KO mice (cGKIfl/fl; Tie2-Cre; n=3). B, Representative Western blots showing that cGKI expression levels in whole brain and heart protein extracts were not different between genotypes. C and D, Intravital microscopy, m. cremaster. Time course of changes in permeability during 20 minutes of continuous local ANP (100 nmol/L) or vehicle and additional 10 minutes of histamine (2 μmol/L) superfusion in control (cGKI+/+; C) and in EC cGKI KO mice (D; n=6 mice per genotype and treatment; *P<0.05 vs vehicle). Right in C and D, Representative original photographs.
by ANP-induced phosphorylation of vasodilator-stimulated phosphoprotein at position Ser239, the cGKI-preferred site; Figure 4B). The specific cGKI inhibitor Rp-8-PET-cGMPs (100 μmol/L; pretreatment during 15 minutes) prevented the stimulatory effects of ANP on vasodilator-stimulated phosphoprotein phosphorylation at Ser239. In contrast, ANP did not induce phosphorylation of vasodilator-stimulated phosphoprotein at Ser157, the site preferred by cAMP-dependent protein kinase (PKA; Figure 4B, top). Together, these data demonstrate that ANP activates GC-A/cGMP/cGKI but not cAMP/PKA signaling in cultured HDMECs.

To study whether ANP influences histamine-induced Rho-A activation, we combined a pull-down assay of activated GTP–bound RhoA13 with Western blot analyses of site-specific phosphorylation of the ROCK substrate myosin–binding subunit of myosin-associated phosphatase type 1 (MYPT1). As shown in Figure 4C and 4D, histamine caused significant Rho-A activation and MYPT1 phosphorylation. ANP did not alter basal or histamine-induced RhoA/ROCK activities (Figure 4C and 4D).

Next, we determined whether ANP instead influences endothelial Ca2+ responses to histamine. Calcium levels were measured in HDMECs loaded with a calcium-sensitive fluorophore. Figure 5A shows that addition of histamine (2 μmol/L) induces a biphasic [Ca2+]i increase, consisting of an initial rapid and transient peak, followed by a sustained plateau phase. Both the peak and plateau [Ca2+]i increases were significantly attenuated by ANP (100 μmol/L, pretreatment during 10 minutes) and also by the membrane-permeant cGKI activator, 8-Br-cGMP (100 μmol/L for 15 minutes; Figure 5A). We conclude that ANP, via GC-A/cGMP/cGKI signaling, inhibits the Ca2+ responses of HDMECs to histamine.

[Ca2+]i Regulatory Protein Inositol 1,4,5-Trisphosphate Receptor I–Associated Protein Is Not Involved in the Antihistaminergic Actions of ANP

Published studies in human umbilical vein endothelial cells indicated that the initial Ca2+ peak response to histamine mainly reflects Ca2+ release from internal stores, whereas the plateau phase is determined by Ca2+ influx from the external medium.14 As mentioned above, both phases were attenuated by ANP or 8-Br-cGMP, suggesting that activation of cGKI modulates the activity of several calcium-regulatory proteins. In smooth muscle cells, cGMP/cGKI-dependent inhibition of Ca2+ release is mediated by the phosphorylation of inositol 1,4,5-trisphosphate receptor I–associated protein (IRAG), which decreases hormone-induced IP3-dependent Ca2+ release and contraction.15 However, in cultured endothelial cells, we did not detect IRAG by reverse transcription polymerase chain reaction (Figure 5B). To study the relevance in vivo, we performed intravital microscopy studies in mice with targeted deletion of exon 3 of IRAG (IRAG−/− mice), which disrupts IRAG expression.15 As shown in Figure I in the online-only Data Supplement, ANP attenuated the hyperpermeability actions of histamine to the same extent in the m. cremaster microcirculation of IRAG−/− and corresponding wild-type mice. Together, these results indicate that IRAG is not involved in endothelial ANP/cGMP/cGKI signaling.

TRPC6 Channels Are Essential for the Endothelial Calcium and Hyperpermeability Responses to Histamine

Members of the TRPC channel family, especially TRPC3 and TRPC6, are putative candidates mediating the reciprocal
TRPC6−/− mice (yet preserved in corresponding wild-type controls). Hence, activation of TRPC6 channels is essential for the endothelial calcium and hyperpermeability actions of histamine (activation)16 and ANP/cGKI (inhibitory)17 on endothelial [Ca2+]i, and permeability. Reverse transcription polymerase chain reaction analyses showed that HMECs express TRPC6 but not TRPC3 mRNA (Figure 5B). Pretreatment of HMECs with the Ca2+-entry blocker SKF96365 (10 μmol/L; 15 minutes) completely abrogated the plateau phase of the histamine-induced [Ca2+]i raise, thus, partly mimicking the effects of ANP (Figure 6A).

To address the functional involvement of TRPC6 channels in the hyperpermeability actions of histamine in vivo, we tested the response in mice with global inactivation of this protein (TRPC6−/− mice).18 As shown in Figure 6B, the characteristic histamine-induced leakage of FITC-dextran was almost completely abolished in cremaster postcapillary venules of TRPC6−/− mice (yet preserved in corresponding wild-type controls). Hence, activation of TRPC6 channels is essential for histamine-induced leakage in the systemic microcirculation.

ANP, Via cGKI, Induces an Inhibitory Phosphorylation of TRPC6 Proteins

In vascular smooth muscle cells, the activation of the NO-cGMP-cGKI pathway inhibits TRPC6 channel activity through phosphorylation of TRPC6 at Thr69.19 To examine whether ANP-stimulated cGKI induces an inhibitory phosphorylation of TRPC6 proteins in endothelial cells, we used a phospho-specific TRPC6 (Thr69) antibody.17 First, phosphorylation of TRPC6 proteins was validated in human embryonic kidney 293 cells coexpressing GC-A and cGKI after incubation with ANP (10 nmol/L; Figure 7A). In contrast, this phosphorylation was not observed in TRPC3-overexpressing human embryonic kidney 293 cells (Figure 7A), confirming that the antibody binds to TRPC6 but not to other channels of this family.17 Unfortunately, this antibody, which has been raised against the murine sequence,17 did not recognize P-TRPC6 in human dermal EC (not shown). Therefore, we complemented these studies with experiments in cultured murine lung ECs (MLECs). As shown in Figure 7A, treatment with ANP significantly increased the phosphorylation of native TRPC6 proteins in wild-type MLECs. The immunoreactive band was almost absent in ANP-treated TRPC6-deficient MLECs. The weak remaining immunoreactive signal is attributable to a mild unspecific crossreaction of the antibody.17 Together, these results suggest that inhibition of TRPC6 channel activity via its cGKI-dependent phosphorylation at residue Thr69 participates in the counteraction of the endothelial calcium and hyperpermeability effects of histamine by ANP.

ANP Attenuates the Hyperpermeability Effect of the TRPC6 Activator Hyperforin

To corroborate the functional involvement of TRPC6 in the antagonistic permeability actions of histamine and ANP in vivo, we tested whether hyperforin, a recently identified activator of TRPC6,20 replicates the hyperpermeability effects of histamine. Indeed, hyperforin suddenly and strongly increased microvascular FITC-dextran leakage in the m. cremaster preparation of wild-type mice, and this response was markedly attenuated in TRPC6−/− mice (Figure 7B). Notably, in the former, ANP pretreatment drastically inhibited the inflammatory effect of hyperforin (Figure 7C).
Effects of histamine (Figure 8B). Notably, this antihistaminergic effect of ANP was lost when cGKI was restricted inactivation of GC-A (EC cGKI KO mice). Our studies in HDMECs are concordant to many published in vitro studies indicating that cGKI is activated by ANP/cGMP in endothelial cells. However, conclusive in vivo studies about the endothelial role of this kinase are missing. Studies in mice with global deletion of cGKI were hampered by their severe systemic phenotype and early lethality. Therefore, to study the significance of cGKI signaling in endothelial barrier functions, here, we generated a new mouse model with conditional, Tie2-Cre–mediated endothelial cell-specific inactivation of cGKI (EC cGKI KO mice). Our studies in these mice clearly demonstrate that cGKI is the immediate target mediating the antihistaminergic effects of GC-A/cGMP in systemic microvascular endothelium.

Activation of endothelial Gαq-coupled histamine (H1) receptors activates phospholipase Cβ and elevates intracellular 

### Discussion

First, this study shows that ANP exhibits protective effects in the systemic microcirculation exposed to activated mast cells or directly to their main inflammatory mediator, histamine. These protective effects of ANP are mediated by the endothelial GC-A receptor and cGMP-dependent activation of cGKI as downstream target. Second, our study reveals that TRPC6 channels are critically involved in the systemic edematous actions of histamine. cGKI-mediated inhibitory phosphorylation of TRPC6 at position Thr69 is likely to participate in the attenuation of the calcium and hyperpermeability actions of histamine by ANP. Finally, pharmacological inhibition of cGMP degradation with the PDE5 inhibitor sildenafil mimicked the protective actions of ANP and this effect was lost in mice with endothelial inactivation of GC-A or cGKI.

Our observations are in line with a recent study showing that systemic, intra-arterial application of ANP attenuated the histamine-induced vascular leak of FITC-dextran. However, this study did not unravel the cell type(s) and intracellular mechanisms targeted by ANP in vivo. The pathophysiology of acute inflammatory processes is complex and involves the interaction of different subsets of inflammatory cells with vascular endothelial and smooth muscle cells. In fact, the protective ANP effects could be mediated by the GC-A receptor on inflammatory cells, such as macrophages or neutrophils. However, as shown here, the antihistaminergic effects of ANP were totally abolished in mice with endothelial deletion of the GC-A receptor, demonstrating a direct endothelial barrier-enhancing (stabilizing) effect of the peptide in the systemic microcirculation.

Which are the immediate and downstream pathways mediating the counterregulation of the endothelial effects of histamine by ANP/GC-A/cGMP? In general, 3 cGMP-modulated proteins (as third messengers) are expressed in endothelial cells: cGMP-stimulated PDE 2, a dual substrate esterase, and cGMP-stimulated PDE 5, which seems to hydrolyze cGMP under resting conditions but also activates phospholipase Cβ2.5 Therefore, to study the significance of cGKI signaling in endothelial barrier functions, we generated a new mouse model with conditional, Tie2-Cre–mediated endothelial cell-specific inactivation of cGKI (EC cGKI KO mice). Our studies in these mice clearly demonstrate that cGKI is the immediate target mediating the antihistaminergic effects of GC-A/cGMP in systemic microvascular endothelium.

Activation of endothelial Gαq-coupled histamine (H1) receptors activates phospholipase Cβ and elevates intracellular
[Ca\(^{2+}\)], which, via Ca\(^{2+}\)/calmodulin, activates myosin light chain kinase, thereby triggering actin-myosin contraction and paracellular hyperpermeability.\(^9\) Independently from this canonical pathway, histamine stimulates RhoA/ROCK-mediated inhibition of MYPT1, which also increases myosin light chain kinase activity.\(^9\) As shown in experiments with HDMECs, ANP, via cGMP/cGKI, attenuated the histamine-induced elevation of [Ca\(^{2+}\)], but did not interfere with the activation of RhoA/ROCK, indicating a specific modulation of classical histamine signaling. How does cGKI modulate intracellular Ca\(^{2+}\) handling? The current knowledge about the modulatory effect of cGKI on [Ca\(^{2+}\)] is mainly derived from studies in nonendothelial cells, such as smooth muscle cells and cardiomyocytes. In these cells, cGKI phosphorylates proteins regulating the release of calcium from intracellular stores (IRAG, phospholamban) as well as membrane channels (directly involved in calcium influx (BK\(_{Ca}\), L-type calcium channels, TRPC3, and TRPC6 channels; reviewed in Reference 26). In human umbilical vein endothelial cells, the initial Ca\(^{2+}\) increase evoked by histamine seems to be mainly determined by IP\(_3\)-receptor–stimulated Ca\(^{2+}\) release from internal stores, whereas the following plateau phase depends on Ca\(^{2+}\) influx from the external medium.\(^9\) In our studies with HDMECs, cGKI activation by ANP or by 8-Br-cGMP attenuated both the histamine-induced initial peak and subsequent plateau [Ca\(^{2+}\)], raising the suggestion that the kinase modulates different endothelial targets involved either in calcium release or in calcium influx. In smooth muscle cells, cGMP/cGKI-dependent inhibition of Ca\(^{2+}\) release is mediated by the phosphorylation of the IRAG, which decreases hormone-induced IP\(_3\)-dependent Ca\(^{2+}\) release and contraction.\(^26\) However, our experiments in vitro (in HDMECs) and in vivo (in IRAG-deficient mice)\(^15\) exclude a role for IRAG as downstream target of ANP/cGKI signaling in endothelial cells. Hence, the exact mechanism(s) mediating the inhibitory effect of ANP on endothelial Ca\(^{2+}\) release remains unclear and will be an issue for our future studies. One possibility is the enhanced sequestration of Ca\(^{2+}\) into intracellular stores by the sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase, which has been shown for autocrine NO/cGMP signaling.\(^27\)

Endothelial Ca\(^{2+}\) influx occurs via the store-operated Ca\(^{2+}\) entry pathway, involving stromal interaction molecule-1 and Orail, but also through channels gated by second messengers like the TRPC channels.\(^16\) Notably, although TRPC channels have been implicated as essential elements mediating endothelial calcium influx and hyperpermeability responses,\(^16\) their role in systemic microvascular endothelial permeability in vivo remains largely unexplored. Recent studies demonstrated that activation of endothelial TRPC6 channels is essential for lung edema induced in mice by ischemia-reperfusion or by platelet-activating factor.\(^20,28\)

Pharmacological experiments in perfused Rana mesenteric vessels indicated that TRPC6 mediates VEGF-induced hyperpermeability.\(^29\) Our present in vivo studies in mice lacking TRPC6 (TRPC6\(^{-/-}\)) extend and strengthen these observations demonstrating for the first time that TRPC6 channels are in fact indispensable for the hyperpermeability responses of the systemic microcirculation to histamine. The proteins Orail and stromal interaction molecule-1 do not contain phosphorylation sites for cGKI.\(^30\) In contrast, TRPC6 channels have been shown to be negatively regulated by cGMP/cGKI, at least in nonendothelial cells, such as cardiomyocytes and human embryonic kidney 293 cells.\(^7,10,31\) Moreover, we showed recently that the GC-A receptor and TRPC6 channels are closely colocalized within a protein complex.\(^32\) Together, the following observations indicate that TRPC6 channels are indeed involved in the ANP counter-regulation of the endothelial calcium and permeability responses to histamine: (1) HDMECs expresses mRNA for TRPC6 but not for TRPC3; (2) SKF96365, an inhibitor
of receptor-mediated Ca\(^{2+}\) entry, mimicked the inhibitory effects of ANP on histamine-induced calcium influx; (3) ANP, via cGKI, stimulated the inhibitory phosphorylation of TRPC6 at the cGKI-preferred site (Thr\(_{69}\)); and (4) ANP potently prevented vascular leakage in response to the TRPC6 activator, hyperforin.

The PDE5 inhibitor sildenafil, which has been largely used for erectile dysfunction and pulmonary hypertension,\(^{35}\) was shown to improve endothelial dysfunction in metabolic diseases, such as type 2 diabetes mellitus and atherosclerosis.\(^{34,35}\) This protective action is primarily attributed to inhibition of PDE5 in vascular smooth muscle cells, which enhances the vasodilator responses to endothelial NO.\(^{35}\) Indeed, as shown here, sildenafil caused significant venodilatation in the mouse m. cremaster, yet this effect was independent of the endothelial GC-A pathway. The novel finding of our study is that sildenafil directly prevents histamine-induced microvascular barrier dysfunction by inhibition of endothelial PDE5, thereby augmenting the protective endothelial actions of endogenous ANP/GC-A/cGMP/cGKI signaling. Atherosclerosis is considered an inflammatory disease, in which the migration of leucocytes through the endothelial wall is a crucial event.\(^{36}\) Our findings indicate that improvement of endothelial barrier functions might be part of the mechanism(s) of action of sildenafil.

Finally, our study also shows that ANP markedly attenuates secretagogue (C48/80)–induced degranulation of perivascular mast cells in the mouse cremaster, an effect that may also contribute to anti-inflammatory actions of the hormone. Contradicting our results, it was reported that ANP itself can activate mast cells isolated from peritoneum or placenta, with the former being mediated via a GC-A/cGMP–independent mechanism(s) of action of sildenafil.\(^{37}\) ANP, via cGKI, stimulated the inhibitory phosphorylation of TRPC6 at the cGKI-preferred site (Thr\(_{69}\)); and (4) ANP potently prevented vascular leakage in response to the TRPC6 activator, hyperforin.

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In conclusion, our study identifies a new regulatory pathway in endothelial cells by which histamine-induced activation of TRPC6 channels is prevented by ANP-induced, GC-A/cGMP/cGKI-mediated inhibitory phosphorylation of these channels. ANP is an endogenous peptide that has been approved for therapeutic treatment of acute myocardial failure.\(^{42}\) Moreover, PDE5 inhibition with sildenafil prevented histamine-induced vascular leakage in wild-type but not in GC-A- or cGKI-deficient mice, demonstrating that this drug enhances the endothelial barrier–protecting actions of endogenous ANP. Together, our results suggest a therapeutic potential of sildenafil or a combination drug treatment exploiting its cooperation with ANP in the treatment of diseases with endothelial barrier dysfunction.

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**Disclosures**

None.

**References**


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**Significance**

Increased permeability of the microvascular endothelium to fluid and proteins is the hallmark of inflammatory diseases. Understanding the regulation of this process is essential for the identification of novel therapies. Here, we found that the hormone atrial natriuretic peptide, via its cyclic GMP-producing guanylyl cyclase-A receptor and activation of cGMP-dependent protein kinase I, counterregulates endothelial Ca2+ increases and paracellular leakage induced by histamine. This anti-inflammatory effect of atrial natriuretic peptide, which is at least in part mediated by an inhibitory phosphorylation of transient receptor potential canonical 6 channels and subsequent attenuation of Ca2+ entry, is enhanced by pharmacological inhibition of phosphodiesterase 5–mediated cGMP degradation with sildenafil. Our results may have important implications for the development of anti-inflammatory therapeutics.
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Supplemental Figure I. IRAG is not involved in the antihistaminergic actions of ANP. Intravital microscopy, m. cremaster. Time course of changes in net integrated optical intensity (IOI; an index of permeability) during 20 min of continuous local ANP (100 nM/L) or vehicle and additional 10 min of histamine superfusion in wildtype (A) and in IRAG<sup>−/−</sup> mice (B) (n = 5 mice per genotype and treatment; *P < 0.05 vs. vehicle). Top panels in A,B: Representative original photographs.
Supplemental Figure II. Endothelial cGKI mediates the antiinflammatory effects of sildenafil. (A,B) Intravital microscopy, m. cremaster. Time course of changes in net integrated optical intensity (IOI; an index of FITC-Dextran permeability) during 30 min of continuous local sildenafil (1 μM) or vehicle and additional 10 min of histamine (2 μM) superfusion in control mice (cGKIfl/fl) (A) and in mice with conditional, endothelial cGKI deletion (EC cGKI KO) (B). Left side: Representative original photographs. All n = 6 mice per genotype and treatment; *P < 0.05 vs. vehicle or baseline.
For the generation of a new mouse model with endothelial (EC)-selective deletion of cGKI, mice with floxed cGKI (1) were mated with Tie2-Cre® mice (2). Mice homozygous for floxed cGKI and heterozygous for the Tie2-Cre transgene (EC cGKI KO) were studied. Floxed cGKI littermates without Cre® served as controls. Mice with EC-restricted deletion of GC-A (EC GC-A KO), systemic deletion of IRAG (IRAG −/−) or of TRPC6 (TRPC6 −/−) and their appropriate control littermates were generated as described previously (3-5). All study mice were males, aged 3 - 5 months. Transgenic and nontransgenic littermates were compared for all mouse lines and in all experiments. The experiments complied with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and were approved by the animal care committee of the University of Würzburg.

**Intravital microscopy of the mouse cremaster microcirculation**

As a measure of microcirculatory endothelial permeability, leakage of FITC dextran (70 kDa) from the intravascular to the extravascular space within the mouse cremaster muscle was analyzed by intravital fluorescence microscopy as described previously (6). Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg BW) and xylazine (10 mg/kg). The depth of anesthesia was checked by ensuring that noxious pinch stimulation (blunt forceps) of the hindpaw, the forepaw and the ear did not evoke any motor reflexes. Body temperature was maintained at 37°C. The cremaster muscle was prepared for intravital microscopy on a custom-made stage and continuously superfused with bicarbonate-buffered saline (pH 7.4, 34°C) of the following composition (in mM): 138 Na+; 6 K+; 2.5 Ca2+; 1.2 Mg2+; 20 HCO3−; 1.2 SO42−, and 1.2 H2PO4−, at a rate of 1.2 ml/min (6). Thereafter, the animal was transferred onto a microscope stage (Olympus, Hamburg, Germany). At the end of the experiment the mice were sacrificed via intravenous ketamine injection.

To assess microvascular permeability of macromolecules, fluorescein isothiocyanate-labeled (FITC-)dextran (Sigma) was dissolved in PBS at a concentration of 10 mg/ml. The free fluorescent dye in the solution was removed by passing through a size exclusion column. After tail vein injection of 0.1 ml FITC-dextran, intravital fluorescence microscopy was performed using a fluorescence filter for FITC for epillumination. The microscopic images were recorded by a charge-coupled device camera and transferred to a computer system for off-line evaluation. Topical application of ANP (100 nM; Bachem) or vehicle was always started 10 min after i.v. administration of FITC-dextran and continued for 30 min. After 20 min of ANP superfusion, the mast cell secretagogue compound 48/80 (5 µg/ml, 10 min (7), histamine (2 µM, 10 min) or the TRPC6 activator hyperforin (10 µM, 5 min (8)) were superfused (all from Sigma). To stain degranulated mast cells, the experiments with C48/80 were finalized by superfusing a 0.001% solution of ruthenium red (Sigma) for 45 min (7).

Quantitative off-line analysis of the microscopic images was performed with the computer-assisted image analysis system Cell-D (Olympus) (6). The observer was blinded to the treatment and genotype. Changes in microvascular permeability were measured using integrated optical intensity (IOI) as an index. Six interstitial areas, immediately adjacent to postcapillary venules, and 6 corresponding intravascular equal areas, were ramlonly selected for IOI analysis. The same preselected areas were observed every 5 or 10 min, as indicated in the results section and corresponding Figures. In the experiments with C48/80 the number of ruthenium red-positive activated (degranulated) mast cells per mm² was quantified by light microscopy.

**Culture of microvascular endothelial cells and cGMP determinations**

We performed our in vitro studies with microvascular lung EC (MLEC (6)) and with human dermal microvascular endothelial cells (HDMEC, Promocell). Endothelial cells from peripheral lung tissue from EC cGKI KO, TRPC6−/− mice and respective controls were isolated and cultured as described (6). After 3 - 4 days, cells were trypsinized and endothelial cells were selected with magnetic beads coated with anti-mouse CD102 antibody (Becton Dickinson). Cultures were grown to confluence and selected a second time before being plated for experiments. Immunocytochemistry with antibodies against three different endothelial markers (VE-cadherin, von Willebrand factor and claudin-5) demonstrated that
after the second selection more than 95% of cultured cells were endothelia. Experiments were performed with MLEC and HDMEC of passage 2 in mitogen-free, serum-reduced Dulbecco's modified Eagle's medium (0.5% fetal calf serum during 3 h prior to experimentation).

To study the effects of ANP on cyclic GMP content of HDMEC, cells seeded in 24-well plates (50000 cells/well) were serum-starved for 2 hours and then stimulated with ANP for 10 min in presence of the non-specific PDE inhibitor 3-isobutyl-1-methylxanthine (0.5 mM IBMX), or the specific PDE5 inhibitor sildenafil (1 μM) (both from Sigma). The incubation medium was aspirated and intracellular cGMP was extracted with ice-cold 70% (v/v) ethanol. The samples were dried and reconstituted for determination of cGMP content by radioimmunoassay (3).

**Experiments with HEK 293 cells**

Human embryonic kidney (HEK)-293 cells stably expressing GC-A were seeded in 6-well plates and transiently cotransfected with cDNAs coding for cGKIα and TRPC3 or TRPC6 channels using FuGENE reagent according to manufacturer’s instructions (Roche) (9). Two days after transfection the cells were serum-starved during 2 h, thererafter treated with ANP (10 nM) or vehicle during 60 min, and finally lysed with RIPA buffer for western blotting (9).

**Western Blot Analysis**

HDMEC, MLEC or transfected HEK 293 cells plated on 6-well dishes were treated as indicated in the results section and were then harvested with RIPA buffer. Extracted proteins were fractionated by 10% SDS-PAGE. Electrophoresis and immunoblotting were performed as described (9). Antibodies were against cGKI, VASP, VASP phosphorylated at the cGKI-preferred site (Ser239) or at the PKA-preferred site (Ser157) (all from Cell Signaling), MYPT phosphorylated at Thr696 (Millipore) and TRPC6 phosphorylated at Thr99, the cGKI preferred site (10). GAPDH was used for loading controls. The blots were developed using the ECL detection system (Amersham-Pharmacia, Freiburg, Germany) and results were quantitated by densitometry (ImageQuant) (9).

**RhoA activation assay**

6 x 10^5 HDMECs were seeded in culture dishes (Ø 6 cm) and expanded for 24 hours in medium containing 10% FCS. Thereafter cells were kept in serum reduced medium (0.5% FCS) for another 24 hours. After incubation without and with ANP (1 μM) for 10 minutes the cells were stimulated with histamine (10 μM) or solvent control for 30 sec. The cells were lysed in ice-cold GST-Fish (11). Activated RhoA-GTP was precipitated with the Rho-binding domain of Rhotekin coupled to glutathione-Sepharose beads (GE Healthcare, Munich, Germany) (11). The amount of activated and total RhoA was then analyzed by immunoblot.

**Measurement of intracellular calcium**

HDMEC were seeded in a 96-well black wall/clear bottom plate, serum starved (1%) for 24 h, and incubated with 100 μl/well of the Hit Hunter Calcium No WashPlus Assay Kit (Discoverex, Fremont, CA, USA) according to the manufacturer's instructions. The cells were pretreated with ANP (100 nM), the membrane permeable cGMP analog 8Br-cGMP (100 μM), or the Ca^{2+} entry blocker SKF96365 (10 μM) or vehicle (saline) during 15 min and thereafter stimulated with histamine (2 μM, 15 min). The fluorescence intensities were recorded every 5 sec for 1000 sec with a SafireII plate reader (Perkin Elmer, Waltham, MA, USA) equipped with a FITC filter.

**RT-PCR**

Total RNA from HDMEC and HUVEC (cells of passage 2 grown to confluency on 6-well plates) was extracted with TRIzol reagent. First strand cDNA was synthesized from 1 μg of total RNA using Superscript II RNase H^{-} reverse transcriptase (Life Technologies Inc.) and random primers. The following primer pairs were used for the amplification of specific cDNA fragments: IRAG-F (5’-CAAGTTGTTGATGAGGAGCGATTC) and IRAG-R (5’TGCTTTGAGAGAGATGAGGAGAA); TRPC3-F (CTGATGAAGGGTGCCAGGAT) and TRPC3-R (TCCAGCACACCCACTACAAAG); TRPC6-F (5’-ACGAGAGCCAGG ACTATCTG) and TRPC6-R (5’-
CATCTTGCTGGAGTTCAGACT; RGS2-F (5'-CGAGAGCGAGAAAGA) and RGS2-R (5'-TTCTCAGAGAAAGGCTTG).

Statistics

Results are presented as the means ± standard error of mean (s.e.m.). The number of experiments (n) is indicated in the Figure legends. P-values were determined by Student’s t-test if allowed or otherwise by ANOVA followed by non-parametric Mann–Whitney U test, with P < 0.05 considered significant.

References for Material & Methods section


