ACCELERATED COMMUNICATION

Atrial Natriuretic Peptide Protects against Histamine-Induced Endothelial Barrier Dysfunction in Vivo

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ABSTRACT
Endothelial barrier dysfunction is a hallmark of many severe pathologies, including sepsis or atherosclerosis. The cardiovascular hormone atrial natriuretic peptide (ANP) has increasingly been suggested to counteract endothelial leakage. Surprisingly, the precise in vivo relevance of these observations has never been evaluated. Thus, we aimed to clarify this issue and, moreover, to identify the permeability-controlling subcellular systems that are targeted by ANP. Histamine was used as an important pro-inflammatory, permeability-increasing stimulus. Measurements of fluorescein isothiocyanate (FITC)-dextran extravasation from venules of the mouse cremaster muscle and rat hematocrit values were performed to judge changes of endothelial permeability in vivo. It is noteworthy that ANP strongly reduced the histamine-evoked endothelial barrier dysfunction in vivo. In vitro, ANP blocked the breakdown of transendothelial electrical resistance (TEER) induced by histamine. Moreover, as judged by immunocytochemistry and Western blot analysis, ANP inhibited changes of vascular endothelial (VE)-cadherin, β-catenin, and p120

Endothelial barrier function is mainly governed by the balance between interendothelial cell adhesion and retraction. Adherens junctions (AJs) are important subcellular structures responsible for endothelial cell-cell attachment, and they represent multiprotein complexes that consist of vascular endothelial (VE)-cadherin, β-catenin, and p120

ABBREVIATIONS: AJ, adherens junction; VE, vascular endothelial; MLC, myosin light chain; ANP, atrial natriuretic peptide; NPR, natriuretic peptide receptor; HUVEC, human umbilical vein endothelial cell; TEER, transendothelial electrical resistance; TNF-α, tumor necrosis factor-α; FITC, fluorescein isothiocyanate; VEGF, vascular endothelial growth factor; cANF, c-atrial natriuretic factor.
The cardiovascular hormone atrial natriuretic peptide (ANP) is secreted by the cardiac atria as a response to an increased plasma volume. In general, ANP binds to the guanylate cyclase-coupled natriuretic peptide receptor (NPR)-A and NPR-C, which lacks guanylate cyclase function. ANP exerts a hypotensive effect by its natriuretic, diuretic, and vasodilating action. The role of ANP as an important regulator of the cardiovascular system is highlighted by the fact that ANP [carperitide (HANP)] has been approved as drug for the treatment of acute heart failure in Japan. However, ANP has been recognized to possess important additional functions beyond blood pressure regulation: ANP is expressed by macrophages and is able to influence these immune cells by attenuating their inflammatory response (Kiemer and Vollmar, 2001). Most importantly, ANP exerts anti-inflammatory properties in the endothelium (Kiemer et al., 2005). Thus, we posed the working hypothesis that ANP could open new therapeutical options for protecting against endothelial barrier dysfunction. In fact, some evidence is given from in vitro and ex vivo experiments that ANP influences an inflammation-increased permeability (Inomata et al., 1987; Lofton et al., 1991; Kiemer et al., 2002a). However, data precisely demonstrating a beneficial effect of administered ANP on inflammation-induced endothelial barrier dysfunction in vivo are lacking. Moreover, data concerning the effect of ANP on subcellular systems that control permeability are missing.

Therefore, the aims of the study were 1) to examine the in vivo potential of ANP as pharmacological agent counteracting endothelial leakage and 2) to investigate the influence of ANP on key regulators of endothelial permeability [i.e., the endothelial cell adhesion (VE-cadherin) and contraction system (MLC)].

Materials and Methods

Measurement of Vascular Permeability in the Mouse Cremaster Muscle in Vivo. Male C57BL/6NCrI mice (Charles River Laboratories, Sulzfeld, Germany) weighing 23 to 25 g were used. All experiments were performed according to the German legislation for the protection of animals. Surgery was performed as described by Baez (1973). Vascular permeability was analyzed according to Hatekeyama et al. (2006). In brief, mice were anesthetized i.p. using a ketamine (Pfizer, Karlsruhe, Germany)/xylazine (Bayer, Leverkusen, Germany) mixture. Fluorescein isothiocyanate-dextran (150 kDa; Sigma-Aldrich, Taufkirchen, Germany), Ringer solution (control), and ANP (bolus sufficient to reach 200 nM plasma concentration) were circulated by means of humidified air streams at 37°C. A custom-built voltage/current clamp unit in combination with a computer-aided evaluation program was used. Bidirectional square current pulses of 50 µA and 200 ms duration were applied across the monolayer every 2 s. The resistance of the monolayer was calculated by Ohm's law from the induced deflection of the transendothelial voltage.

Immunocytochemistry/Histochemistry and Confocal Laser Scanning Fluorescence Microscopy. HUVECs were cultured on collagen-coated 6-well plates or 60-mm dishes. Western blot analysis was performed as described previously (Kiemer et al., 2002a) and cultured in endothelial cell growth medium (Provitro, Berlin, Germany) containing 10% fetal bovine serum (Biochrom, Berlin, Germany). Cells were used for experiments at passages 1 to 3.

Measurement of Transendothelial Electric Resistance (TEER). HUVECs were cultured on collagen-A (Biochrom)-coated Millicell 12-mm PCF inserts (Millipore, Schwalbach, Germany). TEER measurements were performed with an Ussing-type chamber. The incubation fluid (HEPES-buffer containing 10% fetal bovine serum) was circulated by means of humidified air streams at 37°C. A custom-built voltage/current clamp unit in combination with a computer-aided evaluation program was used. Bidirectional square current pulses of 50 µA and 200 ms duration were applied across the monolayer every 2 s. The resistance of the monolayer was calculated by Ohm's law from the induced deflection of the transendothelial voltage.

Measurement of Rat Hematocrit. Male Sprague-Dawley rats (Charles River Laboratories) weighing 190 to 240 g were used. All experiments were performed according to the German legislation for the protection of animals. Rats were anesthetized i.p. using a fentanyl (Jansen-Cilag, Neuss, Germany)/midazolam (Ratiopharm, Ulm, Germany) mixture, and anesthesia was maintained by 1.5% isoflurane (Abbott, Wiesbaden, Germany). Rats were pretreated for 15 min with ANP (bolus sufficient to reach 200 nM plasma concentration) or PBS, followed by histamine (bolus sufficient to reach 1 µM plasma concentration). Reagents were applied into the jugular vein. Thirty minutes after administration of histamine, blood samples were collected via a jugular artery catheter, and hematocrit was determined by centrifugation in hematocrit capillaries.

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were prepared as described previously (Kiemer et al., 2002a) and cultured in endothelial cell growth medium (Provitro, Berlin, Germany) containing 10% fetal bovine serum (Biochrom, Berlin, Germany). Cells were used for experiments at passages 1 to 3.

Results

ANP Protection against an Inflammation-Impaired Endothelial Barrier Function in Vivo. To judge endothelial permeability in vivo, we measured the extravasation of FITC-dextran (150 kDa) via intravital fluorescence microscopy in postcapillary venules of the mouse cremaster muscle. Twenty minutes after intra-arterial application of ANP (bolus sufficient to reach 200 nM plasma concentration), histamine (30 µM) was superfused for 10 min. Histamine evoked a strong leak of FITC-dextran from the blood into the adja-
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Fig. 1. ANP attenuated histamine-induced increase of endothelial permeability in vivo and in vitro. A, extravasation of FITC-dextran (150 kDa) from venules of the mouse cremaster muscle was measured. Top left, mice were pretreated with Ringer’s solution (control and histamine group) or with ANP (intra-arterial bolus injection sufficient to reach 200 nM plasma concentration). After 20 min, histamine (30 μM) was superfused for 10 min (histamine and ANP + histamine group). Data are expressed as mean ± S.E.M. (n = 6). *p ≤ 0.05 versus histamine. Top right, one representative image is shown for each group of treatment (at time point 45 min for control, histamine, and histamine + ANP; at time point 30 min for ANP alone). Videos showing this FITC-dextran extravasation (movie1, control; movie2, histamine; movie3, ANP + histamine) are available as supplemental data. Bottom left, mice were treated with Ringer solution (control group) or with ANP (intra-arterial bolus injection sufficient to reach 200 nM plasma concentration). Data are expressed as mean ± S.E.M. (n = 6). Bottom right, mice were pretreated with Ringer’s solution (histamine group) or with dexamethasone (10 mg/kg of body weight, i.p.) for 2 h. Histamine (30 μM) was superfused for 10 min. Data are expressed as mean ± S.E.M. (n = 2). B, plasma volume changes were determined by measuring hematocrit values. Rats were pretreated with PBS (control) or with ANP (i.v. bolus injection sufficient to reach 200 nM plasma concentration). After 15 min, histamine was applied (i.v. bolus injection sufficient to reach 1 μM plasma concentration). 30 min later, blood samples were taken and hematocrit was measured. Data are expressed as mean ± S.E.M. (n = 3). *p ≤ 0.05 versus histamine. C, transendothelial electrical resistance (TEER) was used to judge changes in endothelial permeability of HUVECs. Left, histamine concentration-dependently decreases TEER values. Middle, ANP (1 μM, 30 min pretreatment) attenuates the histamine-induced decrease of electrical resistance. One representative graph of four independent experiments is shown. Right, statistical analysis of all experiments performed (n = 4). Data are expressed as mean ± S.E.M. *p ≤ 0.05 versus histamine.
cent tissue. ANP clearly abrogated the histamine-induced extravasation (Fig. 1A, top). Movies of this extravasation are presented as supplemental data (movie1, control; movie2, histamine; movie3, ANP + histamine). ANP alone (at least in the observed 20-min pretreatment) seemed to slightly increase basal permeability (please note the different ordinate scales in Fig. 1A), but this effect was statistically not significant (Fig. 1A, bottom left). Moreover, we aimed to appraise the therapeutical impact of ANP by comparing its beneficial effect with that of a strong anti-inflammatory drug. Thus, we treated mice with a high dose of dexamethasone (10 mg/kg i.p., 2 h) before applying histamine (30 nM). The glucocorticoid completely prevented the histamine-induced extravasation of FITC-dextran.

As a second approach for detecting changes of endothelial permeability in vivo, we measured hematocrit levels. Rats were treated with histamine (i.v. bolus sufficient to reach 1 µM plasma concentration), and hematocrit concentration was determined after 30 min. Because of a reduction in plasma volume (i.e., augmented fluid extravasation), histamine evoked a strong hematocrit increase. ANP (bolus injection sufficient to reach 200 nM plasma concentration, 15-min pretreatment) significantly reduced the permeability-increasing effect of histamine (Fig. 1B).

Characterization of the Barrier-Protecting Effect of ANP in Vitro. Data about an influence of ANP on histamine-induced endothelial leakage in vitro are completely lacking. Thus, we first aimed to verify the effect of ANP in HUVECs. To judge permeability changes, TEER was measured. Upon applying histamine, the electrical resistance of a HUVEC monolayer rapidly dropped within seconds and recovered after approximately 10 min. The extent of this effect is dependent on the histamine concentration used: the resistance was lowered to 55% by 10 µM and to 85% by 1 µM histamine (Fig. 1C, left). ANP (1 µM, 30 min pretreatment) attenuated the drop-down of electrical resistance evoked by histamine (Fig. 1C, middle). The statistical analysis of all experiments (n = 4) performed is depicted in Fig. 1C, right. The large

Fig. 2. ANP inhibited the histamine-evoked morphological changes of adherens junctions. HUVECs were left untreated (control) or were treated with histamine (1 µM, 2 min) alone or in combination with ANP (1 µM, 30 min pretreatment) or the NPR-C agonist cANF (1 µM, 30 min pretreatment). The NPR-A/B receptor antagonist HS-142-1 (10 µg/ml) was given 10 min before ANP. Immunocytochemistry and confocal fluorescence microscopy were performed to analyze morphological changes of VE-cadherin (A), β-catenin (B), and p120ctn (C). One representative image of three independent experiments is shown.
variability of the ANP + histamine group expresses the fact that in two of the four experiments, ANP not only attenuated the effect of histamine but also increased the endothelial resistance (i.e., led to a less permeable endothelium), even if compared with the basal resistance under control conditions. In summary, ANP strongly alleviated endothelial barrier dysfunction induced by histamine in vitro. This warranted the usage of this system for the following investigations into the action of ANP on adherens junctions and the contractile machinery.

**ANP Effects on the Histamine-Evoked Changes of Adherens Junction Morphology and the Histamine-Induced VE-Cadherin Tyrosine Phosphorylation.** Histamine (1 μM) led to strong changes of AJ morphology: the VE-cadherin, β-catenin, and p120^crt^ seam, properly built in untreated endothelial cells (control), becomes fringy, indicating an AJ disassembly (i.e., the retraction of the interendothelial VE-cadherin homodimers and/or an intramembranous lateral shift) (Fig. 2, A–C). Endothelial cells treated with ANP alone showed no effect on AJs. Most importantly, ANP (1 μM, 30-min pretreatment) clearly abolished the detrimental effects induced by histamine (Fig. 2, A–C).

To clarify which natriuretic peptide receptor is involved in mediating the beneficial actions of ANP, we treated cells with the NPR-A/B antagonist HS-142-1 (10 μg/ml, 10 min before ANP) and found that the effects on VE-cadherin disassembly were prevented by this inhibitor. The NPR-C receptor agonist cANP (1 μM, 30 min before histamine) was unable to mimic the effects of ANP (Fig. 2A). Compared with NPR-B, NPR-A binds ANP with a much higher affinity. Thus, our results suggest that the action of ANP was mainly transduced by NPR-A. The C-receptor seemed not to be involved.

Phosphorylation of the VE-cadherin Tyr^{731} residue is associated with AJ disassembly and strong endothelial leakage in vitro (Potter et al., 2005). First, we verified that Tyr^{731} was also phosphorylated by histamine in vivo: vessels of the mouse cremaster muscle showed a strong increase of Tyr^{731} phosphorylation induced by histamine (30 μM, 10 min; Fig. 3A) and the same pronounced localization at cell fringes (Fig. 3A, longitudinal vessel section) as in the in vitro situation (Fig. 3B). Most importantly, as shown both by microscopic (Fig. 3B) and by Western blot analysis (Fig. 3C), ANP completely blocked the histamine-induced VE-cadherin Tyr^{731} phosphorylation. ANP alone evoked no alterations of the phosphorylation (Fig. 2B). Our data clearly point toward a protecting effect of ANP on the integrity of endothelial adherens junctions.

**ANP Effects on the Histamine-Evoked Activation of MLC and the Formation of F-Actin Stress Fibers.** The generation of contractile forces (interaction of actin and myosin) is governed by MLC Thr^{18}/Ser^{19}-phosphorylation. Histamine treatment time-dependently led to a strong phosphorylation of MLC, which was analyzed microscopically (Fig. 4A) and by Western blotting (Fig. 4B). Moreover, histamine evoked a strong change in F-actin organization. Although quiescent endothelial cells showed a cortical F-actin localization, histamine induced the formation of long, cell-spanning stress fibers (Fig. 4C). ANP clearly reduced both MLC phosphorylation (Fig. 4, A and B) and F-actin stress fiber formation (Fig. 4C). ANP alone had no effect on these parameters (Fig. 4, A–C). These results indicate that ANP prevented histamine-evoked activation of the endothelial cell contraction system.

Furthermore, we investigated which NP receptor subtype

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**Fig. 3.** ANP blocked histamine-induced VE-cadherin Tyr^{731}-phosphorylation. A, histamine induces phosphorylation of VE-cadherin at Tyr^{731} in vivo. Mice were treated as described in Fig. 1A. Samples of the mouse cremaster muscle were analyzed via immunohistochemistry and confocal fluorescence microscopy. Histamine (30 μM) was superfused for the indicated times. One representative image of three independent experiments is shown. B and C, ANP inhibits histamine-induced VE-cadherin Tyr^{731}-phosphorylation in vitro. HUVECs were left untreated (control) or were treated with histamine (1 μM, 5 min) or ANP (1 μM, 30 min) alone or with ANP (1 μM) 30 min before histamine was applied. The VE-cadherin Tyr^{731}-phosphorylation was analyzed via immunocytochemistry and confocal fluorescence microscopy (B, n = 3) or biochemically via Western blot (C, n = 2).
was involved in mediating these effects. The NPR-A/B inhibitor HS-142-1 (10 μg/ml, 10 min before ANP) blocked the effects of ANP on MLC phosphorylation (Fig. 4A) and stress fiber formation (Fig. 4C). The NPR-C agonist cANF (1 μM, 30 min) was unable to show beneficial effects (Fig. 4, A and C). Thus, NPR-A/B could be regarded as the major receptors for transducing the actions of ANP in our setting.

**Discussion**

Many severe pathologic conditions, such as sepsis or atherosclerosis, are associated with an inflammation-impaired endothelial barrier function leading to an increased plasma extravasation and thus edema formation (Volk and Kox, 2000; Poredos, 2001). Proinflammatory mediators, such as TNF-α or histamine, are involved in the pathogenesis of these disorders and are strong inducers of vascular leakage. Current therapies against an inflammation-evoked barrier dysfunction (e.g., the administration of glucocorticoids or antihistamines) are often insufficient or even fail (van Nieuw Amerongen and van Hinsbergh, 2002). Therefore, new therapeutic options are needed. Strong progress has been made in recent years concerning the mechanisms involved in the regulation of endothelial permeability (Mehta and Malik, 2006). However, substances that counteract an inflammation-induced vascular leakage by specifically influencing these mechanisms are still largely lacking (van Nieuw Amerongen and van Hinsbergh, 2002).

Initially, the physiological action of the cardiovascular hormone ANP (i.e., the reduction of blood pressure) was mainly ascribed to its natriuretic, diuretic, and vasodilating action. However, ANP was also found to increase endothelial permeability (Huxley et al., 1987). This effect was proven to be

![Fig. 4. ANP inhibited histamine-induced MLC2 Thr18/Ser19-phosphorylation and stress fiber formation. A–C, HUVECs were left untreated (control) or were treated with histamine (1 μM, 5 min) alone or in combination with ANP (1 μM, 30 min pretreatment) or the NPR-C agonist cANF (1 μM, 30 min pretreatment). The NPR-A/B receptor antagonist HS142-1 (10 μg/ml) was given 10 min before ANP. MLC2 Thr18/Ser19-phosphorylation and F-actin were analyzed via immunocytochemistry and confocal fluorescence microscopy (A and C). MLC2 Thr18/Ser19-phosphorylation was additionally analyzed via Western blot (B). One representative image of at least three independent experiments is shown.](image-url)
crucial for the long-term control of plasma volume by ANP (Sabrane et al., 2005). Beyond these permeability-increasing effects on quiescent endothelial cells, ANP has increasingly been recognized to possess barrier-protecting actions on an inflammation-activated endothelium. We could demonstrate that ANP attenuates the TNF-α-induced expression of adhesion molecules and monocyte chemoattractant protein-1 by inhibiting NF-κB activation and p38 mitogen-activated protein kinase signaling (Kiemer et al., 2002b; Weber et al., 2003). In this context, we showed that ANP protects against TNF-α-evoked endothelial barrier dysfunction in HUVECs (Kiemer et al., 2002a). ANP was also shown to lower endothelial leakage in vitro induced by the pro-inflammatory stimuli thrombin (Baron et al., 1989) and VEGF (Pedram et al., 2002).

Thus, ANP has commonly been suggested to work as a barrier-protecting agent. Surprisingly, an obvious question remains unanswered precisely: can ANP be used as pharmacological agent to prevent endothelial barrier dysfunction in vivo? This issue is of special interest, because the drug ANP (carperitide) could open new therapeutic opportunities for protecting endothelial barrier function. In the present study, we for the first time show that ANP administered at a pharmacological concentration is able to prevent endothelial leakage in a (histamine-induced) inflammatory setting in vivo. Different aspects of endothelial permeability were used as read-out parameters and were all beneficially influenced by ANP: macromolecular permeability (FITC-dextran extravasation), plasma volume/fluid changes (hematocrit), and electrical resistance (TEER measurement). Compared with the maximal increase of FITC-dextran extravasation induced by histamine (time point 45 min in Fig. 1A), ANP led to a reduction of approximately 65%. Given this pronounced effect, a therapeutic impact of ANP is not unlikely. A complete blockage of the deleterious effect of histamine was observed in the presence of an extraordinarily high dosage of the glucocorticoid dexamethasone, a highly potent anti-inflammatory drug.

Former studies dealing with ANP and vascular permeability served as valuable hints toward an in vivo relevance of ANP as barrier-protecting agent. However, these reports did not convincingly test the hypothesis that administered ANP exerts beneficial effects on endothelial barrier dysfunction in vivo, because they either used ex vivo models or focused on the endogenous ANP system. 1) Three older reports demonstrate that pharmacological concentrations of ANP attenuate changes of pulmonary wet weight induced by toxic agents, such as reactive oxygen metabolites, paraquat, or arachidonic acid in ex vivo models of isolated-perfused lungs from rabbits or guinea pigs (Inomata et al., 1987; Imamura et al., 1988; Lofton et al., 1991). 2) Blockade of endogenous ANP was shown to deteriorate pulmonary edema formation in rats suffering from high-altitude-induced (Irwin et al., 2001) or HCl-evoked (Wakabayashi et al., 1990) pulmonary vascular leakage, whereas mice lacking the major ANP-degrading enzyme neutral endopeptidase were found to be less susceptible for pulmonary leakage (Irwin et al., 2005a). It is noteworthy that Pedram et al. (2002) showed that VEGF-induced vascular leakage is attenuated in ANP-overexpressing mice, whereas these mice are not protected against histamine-evoked leakage. This might be due to the much lower ANP levels in these animals (plasma level: ~40 pM) compared with our setting, in which ANP is exogenously supplied to reach a pharmacological plasma concentration of 200 nM.

Recently, our group could demonstrate that ANP-treated mice (plasma level, ~35 nM) are protected against LPS-induced septic shock (Ladetzki-Baehs et al., 2007). Because endothelial hyperpermeability is an important pathological feature of sepsis, it can be speculated that the barrier-protecting effect of ANP contributes to the beneficial action in the mouse septic shock model. Our results suggest that pharmacological concentrations of ANP show additional, highly valuable effects beyond its action as an endogenous regulator of permeability.

Adherens junctions and the contractile apparatus are key players in the regulation of endothelial permeability. Both the loss of VE-cadherin function and the activation of MLC result in decreased transendothelial electrical resistance (Garcia et al., 1997; van Buul et al., 2005) and increased macromolecular permeability (Garcia et al., 1995; Nwariaku et al., 2002). Studies investigating the action of ANP on these key systems are as yet completely lacking. We provide the first evidence that ANP interacts with these systems, because we showed that ANP attenuated both adherens junction disassembly (morphological changes and Tyr718 phosphorylation of VE-cadherin) and activation of the contractile apparatus (phosphorylation of MLC and rearrangement of F-actin) induced by histamine. Furthermore, we could demonstrate that ANP exerts these effects predominantly via NPR-A. Because this receptor participates particular guanylate cyclases, it can be speculated that the actions of ANP might be mediated via the second messenger cyclic GMP. Our results add further support to the hypothesis that ANP is an endothelium-protecting agent, because it directly counteracts the detrimental effects of proinflammatory mediators on endothelial barrier function.

Few data exist regarding the action of ANP on subcellular systems and its contribution to permeability regulation. We and others could demonstrate that ANP inhibits F-actin stress fiber formation induced by TNF-α (Kiemer et al., 2002a; Irwin et al., 2005b) or VEGF (Pedram et al., 2002). Interestingly, one study reports that ANP influences tight junctions in bovine aortic endothelial cells (Pedram et al., 2002). In contrast to the dense aortic endothelium, the occurrence of tight junctions is limited in the venous endothelium (Ogunrinade et al., 2002), which represents the predominant site of endothelial hyperpermeability and was investigated in the present study.

In summary, we have revealed ANP as a potent endothelial barrier-protecting agent in vivo. Moreover, we have identified adherens junctions and the contractile apparatus as important subcellular systems targeted by ANP. Most importantly, our study highlights ANP as an interesting pharmacological compound opening a new therapeutic option for the prevention of vascular leakage. This warrants further efforts aiming for an expansion of the therapeutic indications of natriuretic peptides.

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