Molecular Pharmacology Fast Forward. Published on April 15, 2008 as doi:10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

Atrial natriuretic peptide protects against histamine-induced endothelial barrier dysfunction *in vivo*

Robert Fürst, Martin F. Bubik, Peter Bihari, Bettina A. Mayer, Alexander G. Khandoga, Florian Hoffmann, Markus Rehberg, Fritz Krombach, Stefan Zahler, and Angelika M. Vollmar

Department of Pharmacy, Pharmaceutical Biology, University of Munich, Germany (R.F., M.F.B., B.A.M., F.H., S.Z., A.M.V.); Institute for Surgical Research, University of Munich, Germany (P.B., A.G.K., M.R., F.K.)

Running title: ANP protects against endothelial barrier dysfunction in vivo

Address all correspondence to:

Robert Fürst, Ph.D.

Department of Pharmacy, Pharmaceutical Biology, University of Munich

Butenandtstr. 5-13, 81377 Munich, Germany

Phone: +49-89-2180-77189; Fax: +49-89-2180-77170

E-mail: robert.fuerst@cup.uni-muenchen.de

Number of text pages: 21

Number of tables: 0

Number of figures: 4

Number of references: 30

Number of words in the *Abstract*: 216

Number of words in the *Introduction*: 441

Number of words in the *Discussion*: 1,140

Nonstandard abbreviations: AJ, adherens junctions; ANP, atrial natriuretic peptide; NPR, natriuretic peptide receptor; VE-cadherin, vascular endothelial-cadherin; MLC myosin light chain; HUVEC, human umbilical vein endothelial cells; MCP, monocyte chemoattractant protein; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase.

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

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 important pro-inflammatory, permeability-increasing stimulus. Measurements of FITC-dextran extravasation from venules of the mouse cremaster muscle and rat hematocrit values were performed to judge changes of endothelial permeability *in vivo*. Importantly, 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 $p120^{ctn}$ morphology, VE-cadherin and myosin light chain 2 (MLC2) phosphorylation, and F-actin stress fiber formation. These changes seem to be predominantly mediated by the natriuretic peptide receptor (NPR)-A, but not by NPR-C. In summary, we revealed ANP as a potent endothelial barrier protecting agent *in vivo* and identified adherens junctions and the contractile apparatus as subcellular systems targeted by ANP. Thus, our study highlights ANP as an interesting pharmacological compound opening new therapeutic options for preventing endothelial leakage.

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

The endothelium crucially participates in the regulation of important physiological functions, such as blood pressure, coagulation, or host defense, and it represents a barrier that controls the passage of cells, macromolecules, and fluid between the blood and the adjacent tissue interstitium. Beyond its physiological role, the endothelium is also involved in pathological conditions: Endothelial barrier dysfunction is a hallmark of inflammatory processes and still poses an important therapeutical challenge, since a causal pharmacological treatment is as yet widely lacking.

Endothelial barrier function is mainly governed by the balance between interendothelial cell adhesion. 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 $p120^{ctn}$. Under inflammatory conditions VE-cadherin junctions disassemble, thus facilitating paracellular passage, and show an increased tyrosine phosphorylation. Endothelial cell retraction is caused by the activation of the contractile machinery, i.e. the interaction between actin and myosin, which is controlled by phosphorylation of the myosin light chain (MLC). These two regulatory systems could be targets of a successful therapeutic principle.

The cardiovascular hormone atrial natriuretic peptide (ANP) is secreted by the cardiac atria as 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. Recently, 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

(Kiemer et al., 2002a; Lofton et al., 1991; Inomata et al., 1987). 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, aim of the study was (i) to examine the *in vivo* potential of ANP as pharmacological agent counteracting endothelial leakage and (ii) to investigate the influence of ANP on key regulators of endothelial permeability, i.e. the endothelial cell adhesion (VE-cadherin) and contraction system (MLC).

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Measurement of vascular permeability in the mouse cremaster muscle *in vivo*. Male C57BL/6NCrl mice (Charles River, Sulzfeld, Germany) with 23-25 g bodyweight were used. All experiments were performed according to the German legislation for the protection of animals. Surgery was performed as described by Baez (Baez, 1973). Vascular permeability was analyzed according to Hatekeyama *et al.* (Hatakeyama et al., 2006). Briefly, 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, AnaSpec/MoBiTec, Göttingen, Germany) were applied into the left femoral artery. 20 min after ANP application, the cremaster was superfused with histamine (30 µM, Sigma-Aldrich) for 10 min. Dexamethasone 21-phosphate (disodium salt, Sigma-Aldrich) was administered *i.p.* (10 mg/kg bodyweight) 2 h before histamine. Postcapillary venules with diameters of 18-30 µm were analyzed. Ten regions of interests (50x50 µm²) in the interstitial tissue (approx. 50 µm distant from the venule) were randomly selected. Intravital microscopic images were recorded with an IMAGO S/N 382KLO345 CCD-camera (TILL Photonics, Gräfelfing, Germany) and subjected to digital image analysis (TILLvisION 4.0, TILL Photonics).

Measurement of rat hematocrit. Male Sprague-Dawley rats (Charles River) with 190-240 g bodyweight 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 pre-treated 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. 30 min 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 previously described (Kiemer et al., 2002a) and cultured in Endothelial Cell Growth Medium (Provitro, Berlin, Germany) containing 10% FBS (Biochrom, Berlin, Germany). Cells were used for experiments at passages 1-3.

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% FBS) was circulated by means of humidified air streams at 37°C. A custom-built voltage/current clamp unit in connection 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 second. The resistance of the monolayer was calculated by Ohm's law from the induced deflection of the transendothelial voltage.

Immunocyto/histochemistry and confocal laser scanning fluorescence microscopy. HUVECs were cultured on collagen-treated µ-Slides (ibidi, Martinsried, Germany). The NPR-A/B antagonist HS-142-1 (Morishita et al., 1991) was kindly provided by Dr. Y. Matsuda, Kyowa Hakko Kogyo Co., Ltd. (Shizuoka, Japan). cANP was from Bachem (Weil am Rhein, Germany). HUVECs and samples of the mouse cremaster muscle (immediately dissected after histamine treatment) were analyzed immunocyto/ histochemically and by confocal fluorescence microscopy as previously described (Fürst et al., 2005). The following antibodies and reagents were used: mouse monoclonal anti-VE-cadherin (Santa Cruz, Heidelberg, Germany), rabbit polyclonal anti-phospho-Tyr^{731}-VE-cadherin (Biosource/Invitrogen, Karlsruhe, Germany), rabbit polyclonal anti-phospho-MLC2 (Thr^{18}/Ser^{19}) (Cell Signaling/New England Biolabs, Frankfurt a. M., Germany), rhodamine phalloidin (Invitrogen, Karlsruhe, Germany), Alexa Fluor 633-linked goat anti-mouse (Invitrogen), and Alexa Fluor 488-linked goat anti-rabbit (Invitrogen).

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

Western blot analysis. HUVEC were cultured in collagen-treated 6-well plates or 60 mm-dishes. Western blot analysis was performed as previously described (Kiemer et al., 2002a). The following antibodies were used: rabbit polyclonal anti-phospho-Tyr^{731}-VE-cadherin (Biosource), mouse monoclonal anti-VE-cadherin (Santa Cruz), rabbit polyclonal anit-phospho-MLC2 (Thr^{18}/Ser^{19}) (Cell Signaling), and MLC2 (Santa Cruz).

Statistical analysis. Statistical analysis was performed with the GraphPad Prism software version 3.03 (GraphPad Software, San Diego, CA). Unpaired *t* test was used to compare two groups. To compare three or more groups, one-way ANOVA followed by Newman-Keuls post hoc test was used.

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

Results

ANP protects 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. 20 min after *i.a.*-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 adjacent tissue. ANP clearly abrogated the histamine-induced extravasation (Figure 1A, upper panel). 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 pre-treatment) seems to slightly increase basal permeability (please note the different ordinate scales in Figure 1A), but this effect is statistically not significant (Figure 1A, lower left panel). Moreover, we aimed to appraise the therapeutical impact of ANP by comparing its beneficial effect to that of a strong anti-inflammatory drug. Thus, we treated mice with a high dose of dexamethasone (*i.p.*, 10 mg/kg, 2 h) before applying histamine (30 µM). 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 was determined after 30 min. Due to a reduction of 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 pre-treatment) significantly reduced the permeability-increasing effect of histamine (Figure 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 human umbilical vein endothelial cells (HUVECs). To judge permeability changes, transendothelial electrical resistance (TEER) was measured. Upon applying histamine, the electrical

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

resistance of a HUVEC monolayer rapidly drops within seconds and recovers after approx. 10 min. The extent of this effect depends on the histamine concentration used: the resistance is lowered to 55% by 10 µM and to 85% by 1 µM histamine (Figure 1C, left). ANP (1 µM, 30 min pre-treatment) attenuates the drop-down of electrical resistance evoked by histamine (Figure 1C, middle). The statistic analysis of all experiments (n = 4) performed is depicted in the right panel of Figure 1C. The large variability of the ANP+histamine group expresses the fact that in 2 of the 4 experiments ANP did not only attenuate the effect of histamine, but even increased the endothelial resistance, i.e. led to a less permeable endothelium, even if compared to the basal resistance under control conditions. In summary, ANP strongly alleviates endothelial barrier dysfunction induced by histamine *in vitro*. This warrants the usage of this system for the following investigations into the action of ANP on adherens junctions and the contractile machinery.

ANP abolishes the histamine-evoked changes of adherens junction morphology and inhibits the histamine-induced VE-cadherin tyrosine phosphorylation. Histamine (1 µM) leads to strong changes of AJ morphology: the VE-cadherin, β-catenin, and $p120^{ctn}$ seam, properly build in untreated endothelial cells (control), becomes fringy, indicating an AJ disassembly, i.e. the retraction of the inter-endothelial VE-cadherin homodimers and/or an intra-membranous lateral shift (Figure 2 A-C). Endothelial cells treated with ANP alone did not show any effect on AJs. Most importantly, ANP (1 µM, 30 min pretreatment) clearly abolishes the detrimental effects induced by histamine (Figure 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 cANF (1 µM, 30 min before histamine) was not able to mimic the effects of ANP (Figure 2A). Compared to NPR-B, NPR-A binds ANP with a much higher affinity. Thus, our results suggest that the action of ANP is mainly transduced by NPR-A. The C-receptor seems 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} is also phosphorylated by

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

histamine *in vivo*: Vessels of the mouse cremaster muscle show a strong increase of Tyr^{731} phosphorylation induced by histamine (30 µM, 10 min, Figure 3A) and the same pronounced localization at cell fringes (Figure 3A, longitudinal vessel section) as in the *in vitro* situation (Figure 3B). Most importantly, as shown both by microscopic (Figure 3B) and by Western blot analysis (Figure 3C), ANP completely blocked the histamine-induced VE-cadherin Tyr^{731} phosphorylation. ANP alone did not evoke any alterations of the phosphorylation (Figure 2B). Our data clearly point towards a protecting effect of ANP on the integrity of endothelial adherens junctions.

ANP reduces the histamine-evoked activation of myosin light chain (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 leads to a strong phosphorylation of MLC, which was analyzed microscopically (Figure 4A) and by Western blotting (Figure 4B). Moreover, histamine evokes a strong change in F-actin organization. While quiescent endothelial cells show a cortical F-actin localization, histamine induces the formation of long, cell-spanning stress fibers (Figure 4C). ANP clearly reduces both MLC phosphorylation (Figure 4, A and B) and F-actin stress fiber formation (Figure 4C). ANP alone had no effect on these parameters (Figure 4, A-C). These results indicate that ANP prevents histamine-evoked activation of the endothelial cell contraction system.

Furthermore, we investigated which NP receptor subtype 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 (Figure 4A) and stress fiber formation (Figure 4C). The NPR-C agonist cANF (1 µM, 30 min) was not able to show beneficial effects (Figure 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 pathologies like 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 therapeutical options are needed. Strong progress has been made in the 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). Recently, this effect was proven to be crucial for the chronic 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 (MCP-1) by inhibiting NF-κB activation and p38 mitogen-activated protein kinase (MAPK) signaling (Weber et al., 2003; Kiemer et al., 2002b). 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).

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

Thus, ANP has commonly been suggested to work as a barrier protecting agent. Surprisingly, an obvious question has as yet not been answered precisely: Can ANP be used as pharmacological agent to prevent endothelial barrier dysfunction *in vivo*? This issue is of special interest, since the drug ANP (carperitide, HANP®) could open new therapeutical options 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 to the maximal increase of FITC-dextran extravasation induced by histamine (time point 45 min in Figure 1A), ANP led to approx. 65% reduction. Due to this pronounced effect, a therapeutical impact of ANP is not unlikely. A complete blockage of the deleterious effect of histamine was observed in the presence of a extraordinary 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 concisely test the hypothesis that administered ANP exerts beneficial effects on endothelial barrier dysfunction *in vivo*, because they (i) either used *ex vivo* models or (ii) focused on the *endogenous* ANP system: (i) Three older reports demonstrate that pharmacological concentrations of ANP attenuate changes of pulmonary wet weight induced by toxic agents like reactive oxygen metabolites, paraquat, or arachidonic acid in *ex vivo* models of isolated-perfused lungs from rabbits or guinea pigs (Lofton et al., 1991; Inomata et al., 1987; Imamura et al., 1988). (ii) 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). Interestingly, Pedram *et al.* showed that VEGF-induced vascular leakage is attenuated in ANP-overexpressing mice, whereas these mice are not protected against histamine-evoked leakage (Pedram et al., 2002). This might

be due to the much lower ANP levels in these animals (plasma level: ~40 pM) compared to 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). Since 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 (Nwariaku et al., 2002; Garcia et al., 1995). Studies investigating the action of ANP on these key systems are as yet completely lacking. We provide for the first time evidence that ANP interacts with these systems, since we showed that ANP attenuates both adherens junction disassembly (morphological changes and Tyr^{731} 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 the natriuretic peptide receptor (NPR)-A. Since this receptors represent particulate guanylate cyclases, it can be speculated that the actions of ANP might be mediated via the second messenger cyclic guanosine monophosphate (cGMP). Our results add further support to the hypothesis that ANP is an endothelium protecting agent, since it directly counteracts the detrimental effects of proinflammatory mediators on endothelial barrier function.

Only few data exist about the action of ANP on subcellular systems contributing 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.

References

Baez S (1973) An Open Cremaster Muscle Preparation for the Study of Blood Vessels by in Vivo Microscopy. *Microvasc Res* **5**:384-394.

Baron DA, Lofton CE, Newman WH and Currie MG (1989) Atriopeptin Inhibition of Thrombin-Mediated Changes in the Morphology and Permeability of Endothelial Monolayers. *Proc Natl Acad Sci U S A* **86**:3394-3398.

Fürst R, Brueckl C, Kuebler WM, Zahler S, Krötz F, Görlach A, Vollmar AM and Kiemer AK (2005) Atrial Natriuretic Peptide Induces Mitogen-Activated Protein Kinase Phosphatase-1 in Human Endothelial Cells Via Rac1 and NAD(P)H Oxidase/Nox2-Activation. *Circ Res* **96**:43-53.

Garcia JG, Davis HW and Patterson CE (1995) Regulation of Endothelial Cell Gap Formation and Barrier Dysfunction: Role of Myosin Light Chain Phosphorylation. *J Cell Physiol* **163**:510-522.

Garcia JG, Schaphorst KL, Shi S, Verin AD, Hart CM, Callahan KS and Patterson CE (1997) Mechanisms of Ionomycin-Induced Endothelial Cell Barrier Dysfunction. *Am J Physiol* **273**:L172-L184.

Hatakeyama T, Pappas PJ, Hobson RW, Boric MP, Sessa WC and Duran WN (2006) Endothelial Nitric Oxide Synthase Regulates Microvascular Hyperpermeability in Vivo. *J Physiol* **574**:275-281.

Huxley VH, Tucker VL, Verburg KM and Freeman RH (1987) Increased Capillary Hydraulic Conductivity Induced by Atrial Natriuretic Peptide. *Circ Res* **60**:304-307.

Imamura T, Ohnuma N, Iwasa F, Furuya M, Hayashi Y, Inomata N, Ishihara T and Noguchi T (1988) Protective Effect of Alpha-Human Atrial Natriuretic Polypeptide (Alpha-HANP) on Chemical-Induced Pulmonary Edema. *Life Sci* **42**:403-414.

Inomata N, Ohnuma N, Furuya M, Hayashi Y, Kanai Y, Ishihara T, Noguchi T and Matsuo H (1987) Alpha-Human Atrial Natriuretic Peptide (Alpha-HANP) Prevents Pulmonary Edema Induced by Arachidonic Acid Treatment in Isolated Perfused Lung From Guinea Pig. *Jpn J Pharmacol* **44**:211-214.

Irwin DC, Patot MT, Tucker A and Bowen R (2005a) Neutral Endopeptidase Null Mice Are Less Susceptible to High Altitude-Induced Pulmonary Vascular Leak. *High Alt Med Biol* **6**:311-319.

Irwin DC, Rhodes J, Baker DC, Nelson SE and Tucker A (2001) Atrial Natriuretic Peptide Blockade Exacerbates High Altitude Pulmonary Edema in Endotoxin-Primed Rats. *High Alt Med Biol* **2**:349-360.

Irwin DC, Tissot van Patot MC, Tucker A and Bowen R (2005b) Direct ANP Inhibition of Hypoxia-Induced Inflammatory Pathways in Pulmonary Microvascular and Macrovascular Endothelial Monolayers. *Am J Physiol Lung Cell Mol Physiol* **288**:L849-L859.

Kiemer AK, Fürst R and Vollmar AM (2005) Vasoprotective Actions of the Atrial Natriuretic Peptide. *Curr Med Chem Cardiovasc Hematol Agents* **3**:11-21.

Kiemer AK and Vollmar AM (2001) The Atrial Natriuretic Peptide Regulates the Production of Inflammatory Mediators in Macrophages. *Ann Rheum Dis* **60 Suppl 3**:iii68-iii70.

Kiemer AK, Weber NC, Fürst R, Bildner N, Kulhanek-Heinze S and Vollmar AM (2002a) Inhibition of P38 MAPK Activation Via Induction of MKP-1: Atrial Natriuretic Peptide Reduces TNF-α-Induced Actin Polymerization and Endothelial Permeability. *Circ Res* **90**:874-881.

Kiemer AK, Weber NC and Vollmar AM (2002b) Induction of IκB: Atrial Natriuretic Peptide As a Regulator of the NF-KB Pathway. *Biochem Biophys Res Commun* **295**:1068-1076.

Ladetzki-Baehs K, Keller M, Kiemer AK, Koch E, Zahler S, Wendel A and Vollmar AM (2007) Atrial Natriuretic Peptide, a Regulator of Nuclear Factor-KappaB Activation in Vivo. *Endocrinology* **148**:332-336.

Lofton CE, Baron DA, Heffner JE, Currie MG and Newman WH (1991) Atrial Natriuretic Peptide Inhibits Oxidant-Induced Increases in Endothelial Permeability. *J Mol Cell Cardiol* **23**:919-927.

Mehta D and Malik AB (2006) Signaling Mechanisms Regulating Endothelial Permeability. *Physiol Rev* **86**:279-367.

Morishita Y, Sano T, Ando K, Saitoh Y, Kase H, Yamada K and Matsuda Y (1991) Microbial Polysaccharide, HS-142-1, Competitively and Selectively Inhibits ANP Binding to Its Guanylyl Cyclase-Containing Receptor. *Biochem Biophys Res Commun* **176**:949-957.

Nwariaku FE, Liu Z, Zhu X, Turnage RH, Sarosi GA and Terada LS (2002) Tyrosine Phosphorylation of Vascular Endothelial Cadherin and the Regulation of Microvascular Permeability. *Surgery* **132**:180-185.

Ogunrinade O, Kameya GT and Truskey GA (2002) Effect of Fluid Shear Stress on the Permeability of the Arterial Endothelium. *Ann Biomed Eng* **30**:430-446.

Pedram A, Razandi M and Levin ER (2002) Deciphering Vascular Endothelial Cell Growth Factor/Vascular Permeability Factor Signaling to Vascular Permeability. Inhibition by Atrial Natriuretic Peptide. *J Biol Chem* **277**:44385-44398.

Poredos P (2001) Endothelial Dysfunction in the Pathogenesis of Atherosclerosis. *Clin Appl Thromb Hemost* **7**:276-280.

Potter MD, Barbero S and Cheresh DA (2005) Tyrosine Phosphorylation of VE-Cadherin Prevents Binding of P120- and Beta-Catenin and Maintains the Cellular Mesenchymal State. *J Biol Chem* **280**:31906-31912.

Sabrane K, Kruse MN, Fabritz L, Zetsche B, Mitko D, Skryabin BV, Zwiener M, Baba HA, Yanagisawa M and Kuhn M (2005) Vascular Endothelium Is Critically Involved in the Hypotensive and Hypovolemic Actions of Atrial Natriuretic Peptide. *J Clin Invest* **115**:1666-1674.

van Buul JD, Anthony EC, Fernandez-Borja M, Burridge K and Hordijk PL (2005) Proline-Rich Tyrosine Kinase 2 (Pyk2) Mediates Vascular Endothelial-Cadherin-Based Cell-Cell Adhesion by Regulating Beta-Catenin Tyrosine Phosphorylation. *J Biol Chem* **280**:21129-21136.

van Nieuw Amerongen GP and van Hinsbergh VW (2002) Targets for Pharmacological Intervention of Endothelial Hyperpermeability and Barrier Function. *Vascul Pharmacol* **39**:257-272.

Volk T and Kox WJ (2000) Endothelium Function in Sepsis. *Inflamm Res* **49**:185-198.

Wakabayashi G, Ueda M, Aikawa N, Naruse M and Abe O (1990) Release of ANP and Its Physiological Role in Pulmonary Injury Due to HCl. *Am J Physiol* **258**:R690-R696.

Weber NC, Blumenthal SB, Hartung T, Vollmar AM and Kiemer AK (2003) ANP Inhibits TNF-Alpha-Induced Endothelial MCP-1 Expression - Involvement of P38 MAPK and MKP-1. *J Leukoc Biol* **74**:932-941.

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. ANP attenuates 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. Upper left panel: Mice were pre-treated with Ringer solution (control and histamine group), or with ANP (*i.a.* 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 ± SEM (n = 6). $^{*}p \leq 0.05$ vs. histamine. Upper right panel: 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. Lower left panel: Mice were treated with Ringer solution (control group) or with ANP (*i.a.* bolus injection sufficient to reach 200 nM plasma concentration). Data are expressed as mean ± SEM (n = 6). Lower right panel: Mice were pre-treated with Ringer solution (histamine group) or with dexamethasone (*i.p.*, 10 mg/kg bodyweight) for 2 h. Histamine (30 µM) was superfused for 10 min. Data are expressed as mean ± SEM (n = 2).

B, Plasma volume changes were determined by measuring hematocrit values. Rats were pre-treated 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 ± SEM (n = 3). $^{*}p \leq 0.05$ vs. histamine.

C, Transendothelial electrical resistance (TEER) was used to judge changes in endothelial permeability of HUVECs. Left panel: Histamine concentration-dependently decreases TEER values. Middle panel: ANP (1 µM, 30 min pre-treatment) attenuates the histamine-induced decrease of electrical resistance. One representative graph out of 4 independent experiments is shown, each. Right panel: Statistical analysis of all experiments performed (n = 4). Data are expressed as mean ± SEM. $^{*}p \leq 0.05$ vs. histamine.

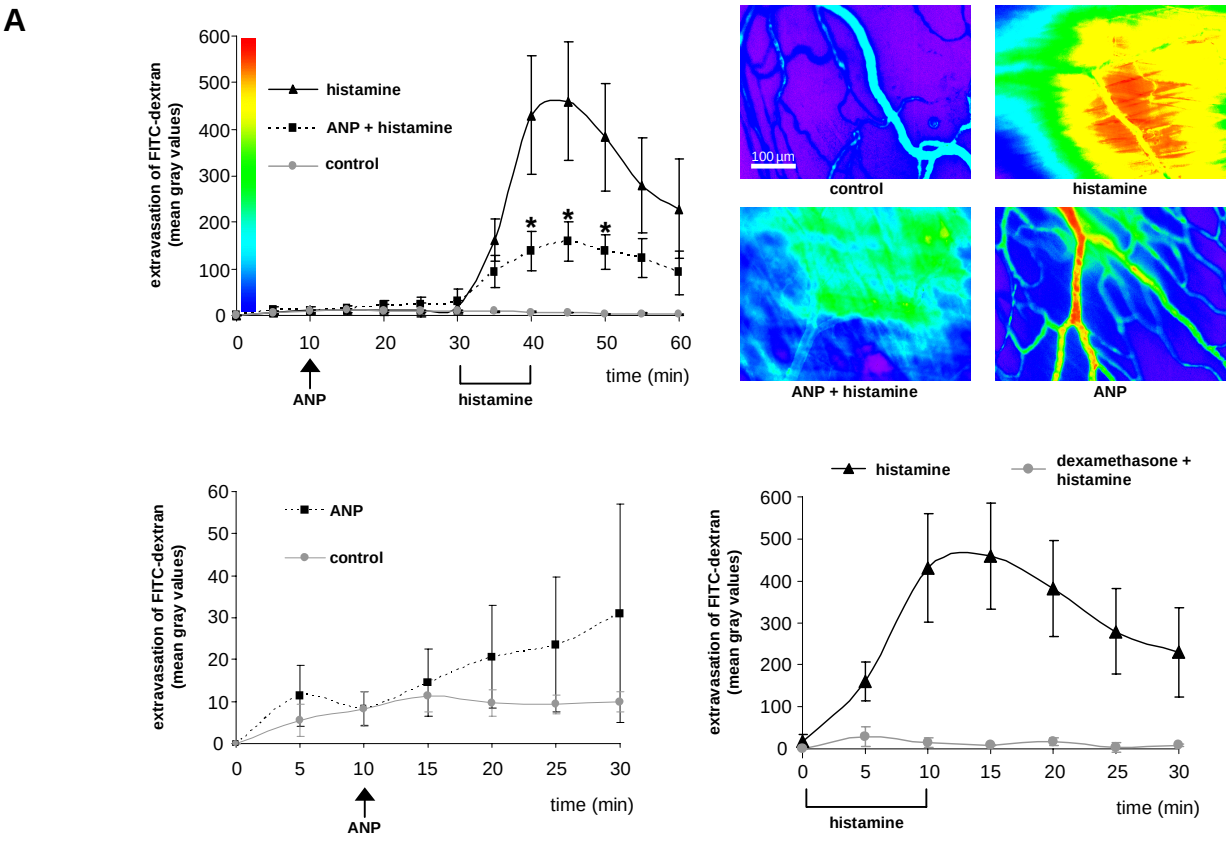
Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

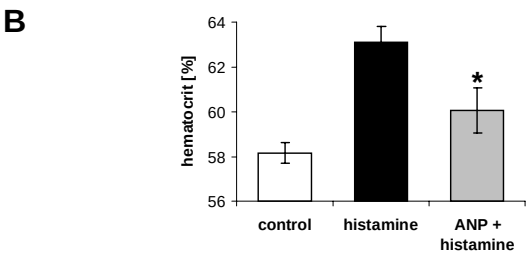
Figure 2. ANP inhibits the histamine-evoked morphological changes of adherens junctions. HUVECs were left untreated (control) or were treated with histamine (1 µM, 2 min) alone or in combination with ANP (1 µM, 30 min pre-treatment) or the NPR-C agonist cANF (1 µM, 30 min pre-treatment). The NPR-A/B receptor antagonist HS142-1 (10 µg/ml) was given 10 min before ANP. Immunocytochemistry and confocal fluorescence microscopy were performed to analyze morphological changes of (A) VE-cadherin, (B) β-catenin, and (C) $p120^{ctn}$. One representative image out of three independent experiments is shown, each.

Figure 3. ANP blocks 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 Figure 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 out of 3 independent experiments is shown. B-C, ANP inhibits histamine-induced VE-cadherin Tyr^{731}-phosphorylation *in vitro*. HUVECs were left untreated (control), 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, $n = 2$).

Figure 4. ANP inhibits histamine-induced MLC2 Thr^{18}/Ser^{19}-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 pre-treatment) or the NPR-C agonist cANF (1 µM, 30 min pre-treatment). The NPR-A/B receptor antagonist HS142-1 (10 µg/ml) was given 10 min before ANP. MLC2 Thr^{18}/Ser^{19}-phosphorylation and F-actin were analyzed via immunocytochemistry and confocal fluorescence microscopy (A, C). MLC2 Thr^{18}/Ser^{19}-phosphorylation was additionally analyzed via Western blot (B). One representative image out of at least three independent experiments is shown, each.

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.





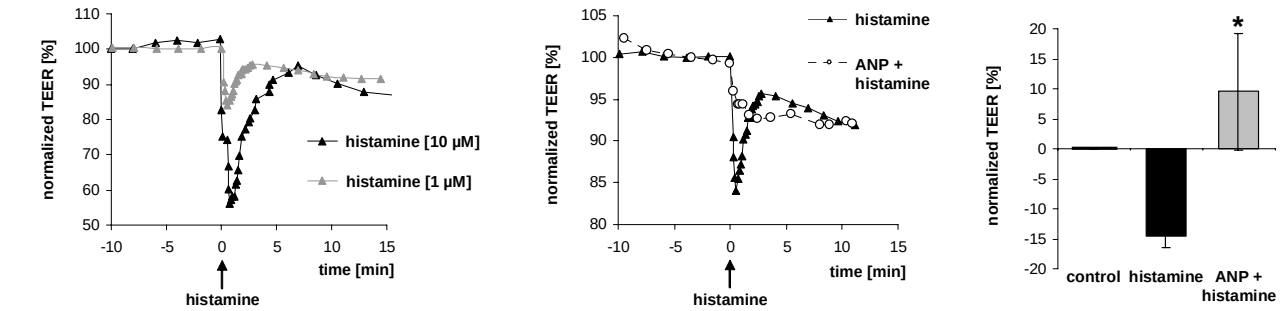


Figure 1

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

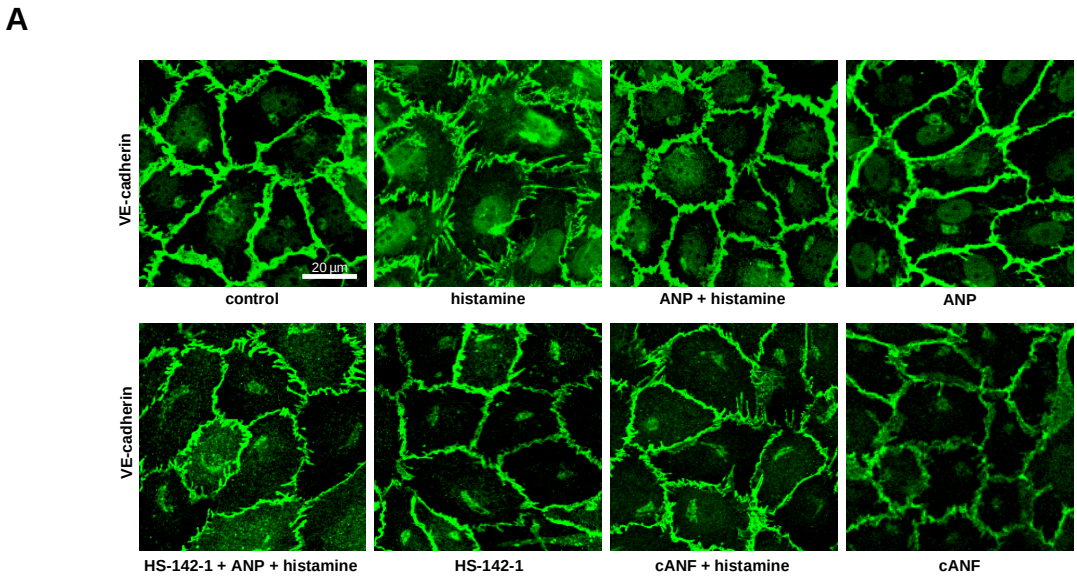


Figure 2

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

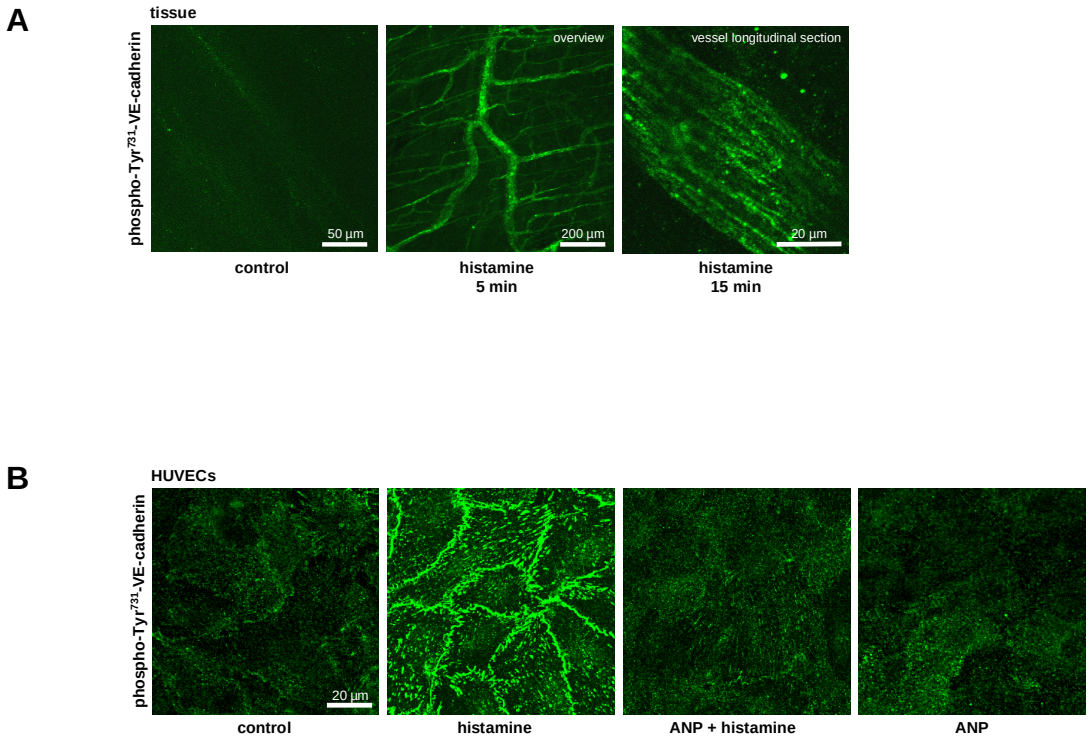
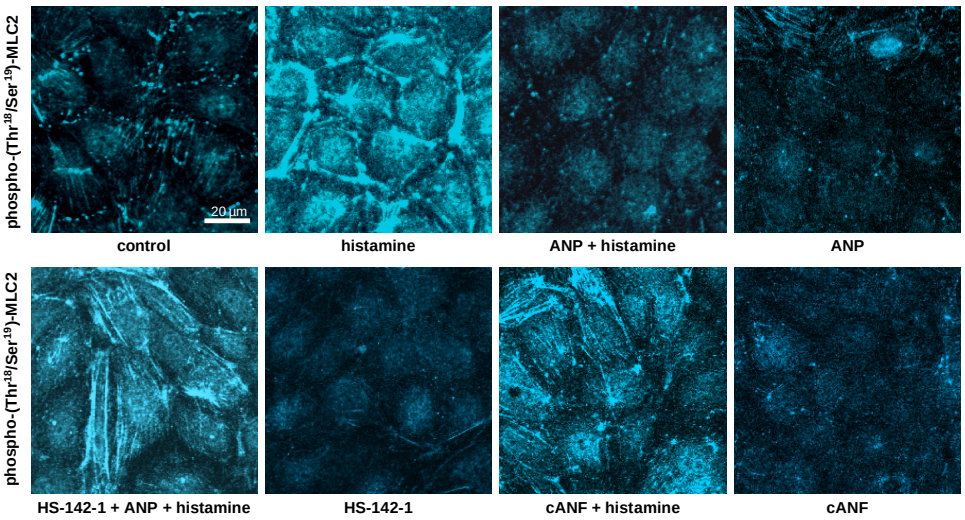


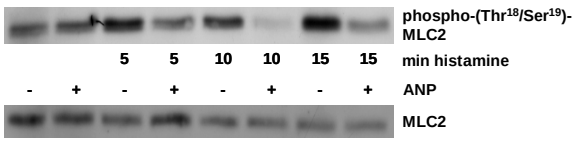
Figure 3

Molecular Pharmacology Fast Forward. Published on April 15, 2008 as DOI: 10.1124/mol.108.045773
This article has not been copyedited and formatted. The final version may differ from this version.

A



B



C

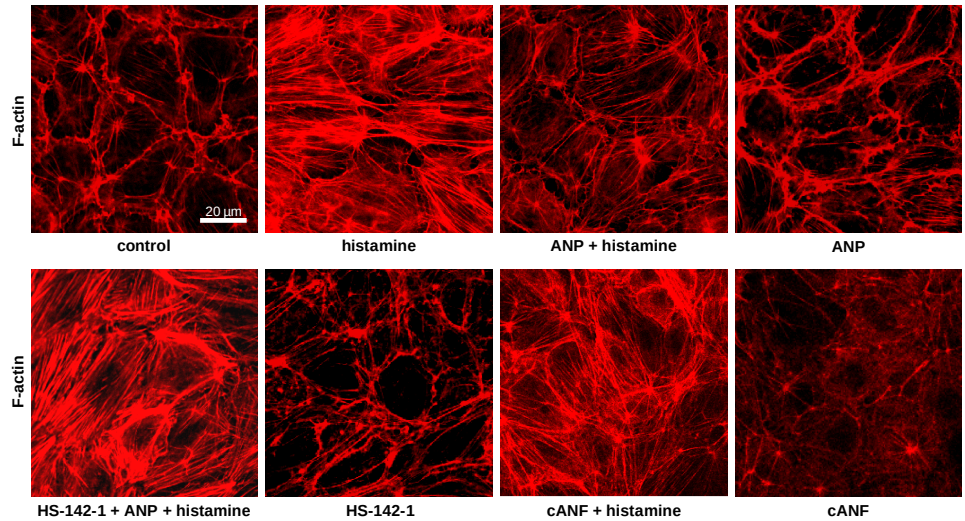


Figure 4