Integrative Physiology

Adrenomedullin Reduces Endothelial Hyperpermeability

Stefan Hippenstiel, Martin Witzenrath, Bernd Schmeck, Andreas Hocke, Mathias Krisp, Matthias Krüll, Joachim Seybold, Werner Seeger, Wolfgang Rascher, Hartwig Schütte, Norbert Suttorp

Abstract—Endothelial hyperpermeability induced by inflammatory mediators is a hallmark of sepsis and adult respiratory distress syndrome. Increased levels of the regulatory peptide adrenomedullin (ADM) have been found in patients with systemic inflammatory response. We analyzed the effect of ADM on the permeability of cultured human umbilical vein endothelial cell (HUVEC) and porcine pulmonary artery endothelial cell monolayers. ADM dose-dependently reduced endothelial hyperpermeability induced by hydrogen peroxide (H$_2$O$_2$), thrombin, and *Escherichia coli* hemolysin. Moreover, ADM pretreatment blocked H$_2$O$_2$-related edema formation in isolated perfused rabbit lungs and increased cAMP levels in lung perfusate. ADM bound specifically to HUVECs and porcine pulmonary artery endothelial cells and increased cellular cAMP levels. Simultaneous inhibition of cAMP-degrading phosphodiesterase isoenzymes 3 and 4 potentiated ADM-dependent cAMP accumulation and synergistically enhanced ADM-dependent reduction of thrombin-induced hyperpermeability. However, ADM showed no effect on endothelial cGMP content, basal intracellular Ca$^{2+}$ levels, or the H$_2$O$_2$-stimulated, thrombin-stimulated, or *Escherichia coli* hemolysin–stimulated Ca$^{2+}$ increase. ADM diminished thrombin- and H$_2$O$_2$-related myosin light chain phosphorylation as well as stimulus-dependent stress fiber formation and gap formation in HUVECs, suggesting that ADM may stabilize the barrier function by cAMP-dependent relaxation of the microfilament system. These findings identify a new function of ADM and point to ADM as a potential interventional agent for the reduction of vascular leakage in sepsis and adult respiratory distress syndrome. (Circ Res. 2002;91:618-625.)

Key Words: adrenomedullin ■ cultured endothelial cells ■ endothelial permeability ■ endothelial barrier dysfunction

The incidence of sepsis and ensuing multiple organ failure has increased over the past two decades and has caused multiple deaths in intensive care units. The development of adult respiratory distress syndrome (ARDS) characterized by noncardiogenic pulmonary edema contributes substantially to a fatal outcome. Increased microvascular permeability is a hallmark of an inflammatory reaction, including ARDS-related pulmonary edema formation. Circumstantial evidence has suggested that endothelial hyperpermeability is related to alterations of the cellular cytoskeleton. Endothelial cells have been shown to contain an elaborate microfilament system allowing active actin- and myosin-based cell contraction. Activation of cell contraction and disturbance of junctional organization subsequently result in the induction of interendothelial gaps followed by enhanced paracellular endothelial permeability. Major initiators of this process are polymorphonuclear leukocyte–derived oxygen metabolites, pore-forming bacterial exotoxins, and endogenous proinflammatory mediators, such as thrombin.

Exposure of endothelial cells to hydrogen peroxide (H$_2$O$_2$) results in the activation of multiple signaling pathways, finally leading to the activation of myosin light chain (MLC) kinase and disrupted barrier function. Thrombin-activated Rho kinase increases the phosphorylation of endothelial cell MLC by directly phosphorylating MLC and simultaneously inhibiting MLC phosphatase, thereby maximally enhancing MLC phosphorylation. The hemolysin of *Escherichia coli* (HlyA) is an important prototype of pore-forming bacterial exotoxins that allows Ca$^{2+}$ influx into the cytosol of target cells. All three stimuli (H$_2$O$_2$, thrombin, and HlyA) have been shown to induce endothelial cell contraction followed by endothelial hyperpermeability in vitro and in vivo.

Previous studies from others and our group have demonstrated an improvement of endothelial barrier function by the activation of adenyl cyclase and/or phosphodiesterase (PDE) inhibition. Analysis of the endothelial cell PDE isoenzyme pattern showed high activities of PDE2 to PDE4. PDE2 mainly metabolizes cGMP, whereas PDE3/4 is specific for cAMP.

Accumulating evidence suggests a pivotal role of adrenomedullin (ADM), a multifunctional regulatory peptide, in sepsis and septic shock. ADM, a 52-amino-acid peptide...

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From Charité, Department of Internal Medicine (S.H., M.W., B.S., A.H., M. Krisp, M. Krüll, J.S., N.S.), Humboldt-University, Berlin, Germany; the Department of Pediatrics (W.R.), University of Erlangen-Nürnberg, Erlangen, Germany; and the Department of Internal Medicine (W.S., H.S.), Justus-Liebig University, Giessen, Germany.

Correspondence to Stefan Hippenstiel, MD, Charité, Department of Internal Medicine/Infectious Diseases, Humboldt-University, Augustenburger Platz 1, 13353 Berlin, Germany. E-mail stefan.hippenstiel@charite.de

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belonging to the calcitonin gene–related peptide family, participates in the control of central body functions, such as vascular tone regulation or fluid and electrolyte homeostasis (see reviews). In systemic inflammatory response, plasma levels of ADM in vertebrates, including human beings, were found to be elevated. Endothelial cells produce ADM in response to proinflammatory cytokines or lipopolysaccharide under the control of the transcription factors nuclear factor for interleukin-6 and activator protein 2.

Calcitonin receptor–like receptor (CRLR) and receptor activity–modifying protein (RAMP)2 and RAMP3 together form ADM receptors coupled to cholela toxin–sensitive G proteins; however, alternative ADM binding receptors may exist. Stimulation of virtually all cells currently investigated with ADM has resulted in a marked increase in cellular cAMP content and subsequent activation of protein kinase A (PKA). In contrast, conflicting results regarding an ADM-induced increase in [Ca\(^{2+}\)] have been reported. Transgenic mice overexpressing ADM in their vasculature have turned out to be resistant to lipopolysaccharide–induced shock, the role of ADM in sepsis deserves special consideration. Moreover, mice with disrupted ADM genes have displayed an extreme hydrops fetalis and cardiovascular abnormalities, suggesting a central role of ADM in the regulation of the cardiovascular system, especially endothelial cell function.

In the present investigation, we tested the hypothesis that elevated ADM levels in systemic inflammatory response stabilize endothelial barrier function by preventing endothelial cell contraction and paracellular fluid flux. ADM treatment diminished endothelial hyperpermeability induced by stimuli as diverse as oxygen metabolites, pore-forming bacterial exotoxins, and endogenous proinflammatory mediators. Moreover, ADM blocked H\(_2\)O\(_2\)-related lung edema formation in a model of isolated rabbit lungs. ADM induced cAMP formation, reduced thrombin- and H\(_2\)O\(_2\)-induced MLC phosphorylation, and prevented endothelial cell contraction. Overall, our data suggest that ADM may act as a counterregulatory peptide in systemic inflammatory response by improvement of endothelial barrier function.

**Materials and Methods**

**Preparation of Endothelial Cells**

Human umbilical vein endothelial cells (HUVECs) and porcine pulmonary artery endothelial cells (PAECs) were prepared as previously described. Human umbilical vein endothelial cells (HUVECs) and porcine pulmonary artery endothelial cells (PAECs) were prepared as previously described. Cells were harvested in lysis buffer containing phosphatase and protease inhibitors. Equal amounts of lysates were subjected to SDS-PAGE (12.5% gel) and blotted. Membranes were simultaneously exposed to goat phospho-specific MLC (Thr18/Ser19) and rabbit extracellular signal–regulated kinase (ERK)1-specific antibodies (both Santa Cruz Biotechnology) incubated with secondary antibodies (IRDye 800–labeled anti-goat and Cy5.5-labeled anti-rabbit, respectively). Proteins were detected by using an Odyssey infrared imaging system (LI-COR Inc).

**Radioligand Binding for ADM**

Competitive receptor binding studies for human ADM were performed with minor modifications as described earlier for neuropeptide Y.

**Determination of HUVEC Cyclic Nucleotide Content**

Cyclic nucleotide content was measured by using a commercially available ELISA (Biotrend).

**Analysis of Endothelial Permeability**

Hydraulic conductivity of endothelial cell monolayers was determined as described previously. Briefly, a confluent cell monolayer on a filter membrane was mounted in a modified chemotaxis chamber, and a hydrostatic pressure of 10\(^2\) mm H\(_2\)O was applied to the “luminal” side of the cell monolayer. The filtration rate across the endothelial monolayer was continuously determined, and the hydraulic conductivity was calculated and expressed as 10\(^{-9}\) cm \(\cdot\) s\(^{-1}\) \(\cdot\) cm H\(_2\)O\(^{-1}\).

**F-Actin Staining**

Cells were fixed in paraformaldehyde and permeabilized and actin was stained by using phalloidin Alexa 488 (Molecular Probes). Cells were analyzed with the use of a Pascal 5 confocal scanning laser microscope (Zeiss).

**Determination of [Ca\(^{2+}\)]**

HUVECs cultured on glass coverslips were loaded with the fluorescent Ca\(^{2+}\)-sensitive dye fura 2 and analyzed by use of a fluorescence spectrophotometer (Instruments S.A.). Excitation wavelength was alternated between 343 and 380 nm. Emitted light was detected at 510 nm. Fura 2 fluorescence was calibrated according to the method described by Grynkiewicz et al.

**Lung Model**

A model of perfused rabbit lungs has previously been described in detail (see overview). Briefly, rabbits were ventilated with room air by use of a Harvard respirator (Hugo Sachs Elektronik). Catheters were placed into the pulmonary artery and left atrium, and the lungs were perfused with Krebs-Henseleit buffer. In parallel, room air ventilation was supplemented with 5% CO\(_2\). Lungs included in the present study had a homogeneous white appearance with no signs of hemostasis, edema, or atelectasis, and they had pulmonary artery and ventilation pressures in the normal range and were isogravimetric during an initial steady-state period of at least 30 minutes.

**Capillary filtration coefficient (Kfc, normalized for wet lung weight) and total vascular compliance were determined gravimetrically, and lung weight gain was calculated. Lungs were perfused for 120 minutes in the absence or presence of 10\(^{-7}\) mol/L ADM. Perfusion (100 \(\mu\)mol H\(_2\)O\(_2\) \(\cdot\) min\(^{-1}\) \(\cdot\) 150 mL\(^{-1}\)) was infused as indicated. In all experiments, 5 \(\mu\)mol/L of thromboxane receptor antagonist BM 13,505 was admixed with the recirculating buffer fluid 10 minutes before stimulus application. Perfusate samples for determination of cAMP were taken after ADM stimulation and were processed for ELISA (Biotrend).

**Statistical Methods**

Depending on the number of groups (A) and the number of different time points studied (B), data of Figures 1, 2, and 6 were analyzed by an A\(\times\)B ANOVA. A one-way ANOVA was used for data of Figures 3 and 4A. Main effects were then compared by an F probability test. A value of P<0.05 was considered significant. Data are displayed as mean±SEM.

An expanded Materials and Methods section can be found in an online data supplement available at [http://www.circresaha.org](http://www.circresaha.org).

**Results**

**ADM Improves Barrier Function of Cultured Endothelial Cell Monolayers**

Sealed PAEC monolayers (Figure 1) and HUVEC monolayers (Figure 2) displayed a hydraulic conductivity of...
HUVECs with 0.1 to 1 hydraulic conductivity of HUVEC monolayers. Incubation of bacterial toxin (Figure 2B). HlyA (0.1 hemolytic units [HU]) abilizing agent, as shown for HUVECs (Figure 2A).

First, we used H₂O₂ to mimic a polymorphonuclear leukocyte-mediated oxidant attack (Figures 1 and 2A). H₂O₂ (1 mmol/L) time-dependently increased the endothelial monolayer permeability of PAECs (Figure 1) and HUVECs (Figure 2A). Pretreatment of PAECs with 0.01 to 1 μmol/L ADM stabilized barrier function of H₂O₂ (10⁻³ mol/L)–exposed endothelial cells. Control monolayers were stable over the entire experimental period. Data are mean±SEM of 4 separate experiments. #P<0.05 vs H₂O₂.

<0.5×10⁻⁵ cm · s⁻¹ · cm H₂O₂⁻¹. The addition of 1 μmol/L ADM as a bolus to PAEC (Figure 1) or HUVEC (Figure 2) monolayers and continuous infusion of 10 μmol/L ADM had no effect on the endothelial barrier function of resting cells within the time frame tested (90 minutes, data not shown).

ADM exposure of HUVECs (1 to 100 μmol/L) had no effect on thrombin-related hyperpermeability, were very effective when used in combination (Figure 2D).

ADM Binds to Endothelial Cells and Induces Formation of cAMP but Does Not Change cGMP or [Ca²⁺]i Levels

Human ¹²⁵I-ADM binds to confluent HUVEC monolayers with a Bₘₐₓ of 0.4 pmol per well and displays a Kₐ of ~147.9 nmol/L. The specificity of ADM binding to HUVECs was confirmed by competition experiments using increasing concentrations of unlabeled ADM. Confluent PAEC cultures showed a Bₘₐₓ of 0.25 pmol per well and a Kₐ of 13.4 nmol/L for human ¹²⁵I-ADM, which could be blocked by increasing concentrations of unlabeled ADM, indicating specificity of peptide binding (see online data supplement).

ADM reduces stimulus-induced phosphorylation of MLCs and alterations of human endothelial cell filamentous actin

ADM (0.1 mmol/L) induced cAMP formation in PAECs (Figure 2B). cAMP elevation with ADM had an effect on thrombin-mediated hyperpermeability (Figure 2D). EDTA treatment of endothelial cells was considered to be a strong signal for endothelial cell retraction followed by endothelial hyperpermeability. ADM exposure of HUVECs (1 to 100 μmol/L) had no effect on basal [Ca²⁺]i content within an observation period of 15 minutes (data not shown). We tested the hypothesis that ADM may reduce the H₂O₂-, thrombin-, or HlyA-mediated rise in [Ca²⁺]i, thereby preventing F-actin rearrangement and hyperpermeability. However, pretreatment of HUVECs with 10 μmol/L ADM before stimulation with H₂O₂, thrombin, or HlyA showed no effect on the [Ca²⁺]i increase induced by these particular agents (see online data supplement).
agents, thereby allowing paracellular fluid flux. Using an antibody directed against phospho-Thr18/phospho-Ser19 of MLC, we demonstrated that ADM reduced thrombin- and H$_2$O$_2$-dependent MLC phosphorylation (Figures 4A and 4B). Simultaneous detection of ERK1 confirmed equal protein loading.

In addition, ADM blocked H$_2$O$_2$-, thrombin-, or HlyA-mediated alterations of endothelial filamentous actin (F-actin), as shown by fluorescence microscopy (Figure 5). HUVECs exposed to solvent (Figure 5A) or 1 nM ADM (Figure 5B) displayed a well-organized peripheral dense band of F-actin with only a few stress fibers. After stimulation of endothelial cells with 1 mmol/L H$_2$O$_2$ for 30 minutes (Figure 5C), 1 U thrombin for 15 minutes (Figure 5E), or 0.1 HU HlyA for 15 minutes (Figure 5G), the amount and density of stress fibers increased, whereas the peripheral dense band was disrupted, and gaps between the endothelial cells were opened. In contrast, in cells pretreated with 1 nM ADM 15 minutes before stimulation with these agents (Figures 5D, 5F, and 5H), F-actin distribution remained unchanged, and intercellular gaps were closed as in the control monolayers.

**Human ADM Stimulates cAMP Formation and Reduces H$_2$O$_2$-Induced Vascular Hyperpermeability in Isolated Rabbit Lungs**

We made use of isolated, ventilated, blood-free perfused rabbit lungs stimulated with H$_2$O$_2$ (100 μmol/min admixed to the 150 mL perfusate over 15 minutes) to analyze the power of ADM in the regulation of endothelial barrier function in a more integrated model (Figure 6). In lungs exposed to H$_2$O$_2$ alone, Kfc increased up to 9.0±2.08 within 45 minutes, followed by massive pulmonary edema formation (Figure 6A). In contrast, ADM (0.1 μmol/L 15 minutes before H$_2$O$_2$ admixture) almost completely prevented H$_2$O$_2$-induced edema formation (Figure 6A). The vascular compliance remained unchanged and was not different between the experimental groups. Pulmonary artery pressure showed some minor elevation on H$_2$O$_2$ infusion but did not display a significant difference between ADM-treated and
-untreated lungs at the time points of the Kf determination (see online data supplement). Moreover, only negligible changes (≤1 mm Hg) of microvascular pressures were observed in both experimental groups, indicating that the reduced edema formation observed in the ADM-stimulated rabbit lungs was not due to reduced filtration pressure (see online data supplement).

cAMP content in the perfusate collected from the rabbit lungs processed for analysis of Kf was strongly increased in ADM-exposed lungs (Figure 6B).

Discussion

Recent studies have demonstrated elevated plasma levels of ADM in vertebrates with a systemic inflammatory response. However, the role of ADM in the complex and dynamic disease process of sepsis is still largely undefined. On the one hand, the high ADM plasma levels observed in septic humans may contribute to hypotension and hypodynamic circulatory response in sepsis, thereby contributing to disease progress. On the other hand, transgenic mice overexpressing ADM in their vasculature turned out to be resistant against lipopolysaccharide-induced shock, suggesting a rather beneficial effect of elevated ADM levels in sepsis.

Considering that endothelial hyperpermeability is the hallmark of an inflammatory reaction, and that mice lacking a functional ADM gene displayed an extreme hydrops fetalis, we tested the hypothesis that elevated ADM levels stabilized endothelial barrier function. ADM preincubation reduced endothelial barrier dysfunction in ADM. H2O2, released by polymorphonuclear granulocytes typical permeability-inducing agents to investigate the effects of ADM. H2O2, released by polymorphonuclear granulocytes in inflammatory reactions, activates complex signaling pathways in endothelial cells, including active myosin-based cell contraction and protein kinase C (PKC) activation. As a prototype of a bacterial pore-forming exotoxin, allows Ca2+ influx into the cytosol according to the transmembrane Ca2+ gradient and potently induces NO production as well as the expression of endothelial cell adhesion molecules.

All three stimuli induced active actin-myosin–based cell contraction, thereby increasing enhanced paracellular endothelial permeability. Notably, ADM preexposure of endothelial cells increased intracellular cAMP and reduced MLC phosphorylation, thereby stabilizing the endothelial cell microfilament system.

Figure 4. ADM reduced Thr- and H2O2-related MLC phosphorylation in HUVECs. Preincubation of cells with 1 μmol/L ADM reduced Thr (0.1 U/mL)- and H2O2 (1 mmol/L)-related MLC phosphorylation as shown by Western blot with use of MLC-phospho-Thr18/Ser19–specific antibody (A and B). Phosphorylated MLC (P-MLC) and ERK1 were simultaneously detected by an infrared imaging system. MLC phosphorylation was normalized to detected protein of ERK1; total amount is demonstrated in relation to Thr- or H2O2-induced MLC phosphorylation (A). Data presented in panel A are mean ± SEM of 3 separate experiments. #P<0.05 vs control; *P<0.05 vs ADM; §P<0.05 vs Zarda. For panel B, *P<0.05 vs control; #P<0.05 vs ADM and vs Zarda; and *P<0.05 vs ADM.
lial cell monolayers greatly reduced the increase in hydraulic conductivity in response to all three agents. It has been suggested that ADM-mediated cAMP elevation contributes to ADM effects in the vasculature. In line with previous studies, ADM incubation increased cAMP content in cultured endothelial cells. Human and porcine endothelial cells contain PDE3 and PDE4 for the degradation of cAMP and PDE2 for the degradation of cGMP. Inhibition of PDE3/4 with the dual-selective PDE3/4 inhibitor zardaverine significantly increased ADM-related cAMP accumulation and strengthened the endothelial barrier–protective effect of ADM as assessed for thrombin-related hyperpermeability. ADM seems to be as potent as a pharmacological stimulator of cAMP elevation, regarding maintenance of endothelial barrier function. Moreover, ADM treatment elevated cAMP levels in isolatedperfused rabbit lungs and reduced H2O2-related edema formation. This is consistent with previous studies showing that cAMP elevation potently blocks hyperpermeability in isolated rabbit lungs.

To exclude the effects of H2O2-dependent pulmonary vasoconstriction on edema formation, we used the thromboxane receptor antagonist BM 13.505. Although ADM was known to reduce systemic blood pressure and, in some systems, pulmonary hypertension, no substantial changes in pulmonary artery perfusion pressure were noted in H2O2- or solvent-exposed rabbit lungs pretreated with BM 13.505.

In line with the presently described role of ADM in the regulation of endothelial permeability under inflammatory conditions, recent observations in mice lacking a functional ADM gene point to a general role of ADM in permeability regulation: mice lacking a functional ADM gene displayed an extreme hydrops fetalis as well as cardiovascular abnormalities and died at mid gestation. Overall, these observations suggest a pivotal role of ADM in the regulation of endothelial barrier function.

Alterations of the endothelial cell microfilament system are accompanied by active actin-myosin–based cell contraction, allowing increased paracellular fluid flux, which seems to be critical for edema formation under inflammatory conditions. ADM reduced thrombin- and H2O2-related phosphorylation of MLC and blocked endothelial cell contraction, intercellular gap formation, and stress fiber formation. Besides classic Ca2+/calmodulin-dependent MLC kinases, Rho kinase may contribute to thrombin-related MLC phosphorylation. Although MLC kinase phosphorylates MLC at Ser19 and Thr18 as a sole mode of action, Rho kinase additionally blocks myosin phosphatase type 1, thereby enhancing MLC phosphorylation. Inasmuch as cAMP elevation reduced Rho kinase–dependent phosphorylation of MLC in lipopolysaccharide-exposed endothelial cells and
Rho kinase was identified as a central regulator of thrombin-induced endothelial cell contraction. ADM-related increased cAMP may act via inhibition of the Rho–Rho kinase pathway. Moreover, PKC-dependent phosphorylations of MLC and important permeability-regulating functional proteins, such as vasodilator-stimulated phosphoprotein, zona occludens protein-1, and vascular endothelial cadherin, significantly contribute to barrier dysfunction. Although cAMP elevation seems not to prevent stimulus-dependent PKC activation in endothelial cells, cAMP-related PKA activation may counterregulate PKC-induced phosphorylation effects. For example, it has been shown that PKA-dependent phosphorylation of the Ser157 residue of vasodilator-stimulated phosphoprotein diminishes paracellular permeability through the relaxation of actin cytoskeletal tension.

The data presented support the notion that ADM binds via specific receptors to endothelial cells. However, the situation is complex inasmuch as CRLR and RAMP2 and RAMP3 together form ADM receptors. Moreover, alternative ADM binding sites may exist. Interestingly, the expression of CRLR-RAMP2/CRLR-RAMP3 complexes apparently undergoes regulatory changes in different tissues and stages during sepsis, thereby contributing to ADM-related effects in systemic inflammation.

Besides the widely accepted ADM-dependent cAMP elevation, conflicting results were reported regarding an ADM-induced rise in [Ca\(^{2+}\)]\(_{i}\) in endothelial cells. In the present study, ADM exposure had no effect on [Ca\(^{2+}\)]\(_{i}\) levels in cultured HUVECs and showed no modulation of the H\(_2\)O\(_2\)-, HlyA-, or thrombin-mediated increase in [Ca\(^{2+}\)]\(_{i}\]. In addition, ADM-treated endothelial cells displayed no changes of intracellular cGMP content, even in cells with PDE2 inhibition to block cGMP degradation.

In summary, the data presented indicate that specific binding of ADM to endothelial cells elevated cAMP levels, blocked H\(_2\)O\(_2\)- and thrombin-related MLC-phosphorylation, and prevented endothelial cell contraction. ADM markedly reduced thrombin-, HlyA-, and H\(_2\)O\(_2\)-related endothelial hyperpermeability. Simultaneous inhibition of cAMP-degrading pathways, cAMP accumulation and Ca\(^{2+}\) increase in endothelial cell function. Lab Invest. 1992;67:24–30.


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ADRENOMEDULLIN REDUCES ENDOTHELIAL HYPERPERMEABILITY

Stefan Hippenstiel¹, Martin Witzenrath¹, Bernd Schmeck¹, Andreas Hocke¹, Mathias Krisp¹,
Matthias Krüll¹, Joachim Seybold¹, Werner Seeger³, Wolfgang Rascher³, Hartwig Schütte³,
and Norbert Suttrop¹

1) Charité, Department of Internal Medicine, Humboldt-University, 13353 Berlin, Germany;
2) Department of Pediatrics, University of Erlangen-Nürnberg, 91054, Erlangen, Germany;
3) Department of Internal Medicine, Justus-Liebig University, 35392 Giessen, Germany;
MATERIAL AND METHODS:

Materials: Tissue culture plastic ware was obtained from Becton-Dickinson, Heidelberg, Germany. Medium 199, fetal calf serum (FCS), Hanks' balanced salt solution (HBSS), phosphate buffered saline (PBS), trypsin-EDTA-solution, Puck's A, HEPES and antibiotics were from Gibco, Karlsruhe, Germany. Collagenase (CLS type II) was purchased from Worthington Biochemical Corp., Freehold, N.J.. Gelatin from porcine skin type I, glutaraldehyde grade II, sodium nitroprusside, thrombin, and adrenomedullin were purchased from Sigma Chem. Co., Munich, Germany. Sterile Krebs-Henseleit-buffer containing 120 mmol/L NaCl, 4.3 mmol/L KCl, 1.1 mmol/L KH₂PO₄, 24 mmol/L NaHCO₃, 2.4 mmol/L CaCl₂, 1.3 mmol/L MgCl₂, as well as 2.4 g/L glucose was prepared by Serag-Wiessner (Naila, Germany). The thromboxane receptor antagonist BM 13.505 (4-[2-(4-chlorbenzolsulfonylamino)-ethyl]-phenyl acetic acid) was a generous gift from Boehringer AG (Mannheim, Germany). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was a kind gift of T. Podzuweit, Max Planck Institute for Physiology (Bad Nauheim, Germany). 6-(Difluormethoxy-3-methoxyphenyl)-3-(2H)-pyridazone (zardaverine) was synthesized by Byk Gulden (Konstanz, Germany). Polycarbonate micropore filter membranes (25 mm diameter, 5 μm pore size) were purchased from Nucleopore GmbH, Tübingen, Germany. Unlabeled adrenomedullin was from Bachem Biochemica GmbH, Heidelberg, Germany, and ¹²⁵I-labeled adrenomedullin (specific activity 2000 Ci/mmol) was obtained from Anawa Laboratories AG, Zürich, Switzerland. All other chemicals used were analytical grade and obtained from commercial sources.

Preparation of endothelial cells: Human umbilical cord vein endothelial cells (HUVEC) were isolated from umbilical cord veins and identified as previously described. Briefly, cells obtained from collagenase digestion were washed, resuspended, cultivated in MCDB 131-10% FCS and seeded into tissue culture flasks (80 cm²) or 24-well plates (Becton-Dickinson, Heidelberg, Germany). Porcine endothelial cell monolayers (PAEC) were prepared from pulmonary arteries of freshly slaughtered pigs by exposure to 0.1 % collagenase in Puck's saline
for 12-15 min at 37°C. Cells were dispersed, characterized, and maintained in medium M 199/10 % FCS in a humified atmosphere (37°C, 5 % CO₂) as previously described. Studies were performed using confluent porcine endothelial cell monolayers in their third passage.

**Radioligand binding for adrenomedullin:** Competitive receptor binding studies for human ADM were performed similar as described earlier for neuropeptide Y. Briefly, confluent cultures of PAEC or HUVEC grown on 24-well plates were washed twice and incubated in assay buffer containing 10 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L KHPO₄, 1.2 mmol/L MgSO₄, 25 mmol/L NaHCO₃, 10 mg/ml bovine serum albumin, 0.5 µg/ml bacitracin, 0.5 µg/ml soybean trypsin inhibitor at a pH of 7.4. For radioligand binding studies 10⁷ CPM ¹²⁵I-labeled human ADM (specific activity 2000 Ci/mmol) per ml and varying concentrations of unlabeled ADM reaching from 0.5 nmol/L to 1 µmol/L (for PAEC) and from 8 nmol/L to 1 µmol/L (for HUVEC) were applied. Cells were incubated for 90 min at 20°C. Thereafter, assay buffer was removed and subsequently wells were washed with ice-cold assay buffer and ice-cold PBS. Cells were lysed by addition of lysis buffer containing 3 mol/L acetic acid and 8 mol/L urea and remaining radioactivity was quantified in a γ-counter. Non-specific binding was defined as ¹²⁵I ADM binding in the presence of 1 µM unlabeled peptide. Maximal binding was calculated from specifically bound ADM by using PRIZM Version 2.0, GraphPad Software, San Diego, USA. Data were also plotted according to Scatchard.

**Determination of HUVEC cyclic nucleotide content:** Endothelial cell cyclic nucleotide content was measured by using commercially available enzyme-linked immunosorbent assay (ELISA) (Biotrend, Cologne, Germany). Briefly, HUVEC or PAEC cultured in 24-wells were
washed, and stimulated at 37°C as indicated. Incubation was stopped by addition of ice-cold buffer and rapid freezing. Cells were lysed by using 0.1 mol/L HCl, lysates were collected and centrifuged to remove cell debris. Aliquots of the extracts and standards were acetylated by addition of acetic anhydride and triethylamine (1/2 v/v) and processed for ELISA according to the manufactures instructions.

Analysis of endothelial permeability: Hydraulic conductivity of endothelial cell monolayers grown on polycarbonate filter membranes was determined as described previously. Briefly, a confluent cell monolayer on a filter membrane was mounted in a modified chemotaxis chamber and a hydrostatic pressure of 10 mm$^3$ $H_2O$ was applied to the “luminal" side of the cell monolayer. The filtration rate across the endothelial monolayer was continuously determined and the hydraulic conductivity was calculated and expressed as $10^5$ cm x s$^{-1}$ x cm $H_2O^{-1}$.

Experimental protocol: Only monolayers which showed a final hydraulic conductivity of less than $0.5 \times 10^{-5}$ cm x s$^{-1}$ x mm$^3$ $H_2O^{-1}$ in the presence of a hydrostatic pressure of 10 mm$^3$ $H_2O$ were used (“sealed" filters, see reference$^4$ for details). ADM, zardaverine, thrombin, HlyA, or $H_2O_2$ were added as a bolus into the upper compartment at the time point indicated. $H_2O_2$ and zardaverine were also added to the fluid reservoir which provided the hydrostatic pressure to the upper compartment. Thus, fluid filtrated from the upper into the lower compartment was replaced by $H_2O_2$ or zardaverine-containing buffer, respectively, from the reservoir.
**F-actin staining:** HUVEC were grown on Thermanox® slides (Nunc, Wiesbaden, Germany). After stimulation, slides were fixed for 1 h in 3% paraformaldehyde at room temperature for 5 min and rinsed three times in PBS. Permeabilization of cell membranes was performed using 0.1% Triton X100 for 5 min followed by three wash steps with PBS. F-actin was stained with phalloidin Alexa 488 (Molecular Probes, Eugene, OR) (1:400) for 30 min. After washing with PBS, Thermanox® slides were applied up-side-up on glass slides and covered with Fluoromount-G™ (Electron Microscopy Sciences, PA) mounting medium by a congruent glass coverslip. Nailpolish was used for slide sealing. Slides were analyzed using the Pascal 5 confocal scanning laser microscope (Zeiss, Jena, Germany) equipped with an air-cooled argon laser (Axioskop 2 Mot microscope, Zeiss). Alexa 488 fluorescence was excited with 488 nm argon-ion laser line and imaged using a NT80/20/488 beamsplitter and a 505-nm longpass emission filter. All images were taken with a 63x 1.4 NA Plan-Apochromat III DIC objective with image resolution 1024x1024 pixels.

**Phosphorylation of myosin light chain:** Endothelial cells were stimulated as indicated, and washed three times with ice-cold PBS containing NaF 100 mmol/L, Na₃VO₄ 2 mmol/L, Na₄P₂O₇ 15 mmol/L. Cells were harvested by scraping in lysis-buffer (Tris-HCl, 50 mmol/L, pH 7.4, EDTA 0.25 mmol/L, PMSF 1 mmol/L, NP 40 1% (v/v), and protease inhibitors Antipain, Leupeptin, and Pepstatin, all 10 μg/ml). Lysates were aligned for protein content and equal amounts were subjected to SDS-PAGE (12.5% gel) and blotted on nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were blocked using blocking buffer (LI-COR Inc. Lincoln, NE) diluted in PBS (1/1 v/v) for 1 h. Membranes were simultaneously exposed to goat phospho-MLC (Thr 18/Ser 19) (1:200)- and rabbit ERK2-
specific antibody (1:1000) (both Santa Cruz Biotechnology, Heidelberg, Germany) in blocking buffer containing 0.1% Tween-20 (v/v) overnight at 4°C. After washing in PBS, membranes were incubated with secondary antibodies (IRDye 800-labeled anti-goat 1:2500, Cy5.5-labeled anti-rabbit 1:2500, respectively) in blocking buffer for 45 min at room temperature. After extensive washing in PBS, membranes were analyzed using Odyssey™ infrared imaging system (LI-COR Inc. Lincoln, NE).

**Determination of intracellular Ca²⁺-levels [Ca²⁺]i**: HUVEC cultured on fibronectin-covered glass cover slips were loaded with the fluorescent Ca²⁺-sensitive dye fura-2 by incubation with 0.025% (w/v) pluronic F-127 for 30 min and 3 μmol/L fura 2-AM at 37°C. Cells were washed with modified Tyrode’s solution containing 132 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, 5 mmol/L glucose, and 9.5 mmol/L HEPES. Thereafter, cover slips were mounted into a fluorescence spectrophotometer (Instruments S. A., Munich, Germany). Excitation wavelength was alternated between 343 and 380 nm. Emitted light was detected at 510 nm. Fura 2 fluorescence was calibrated according to the method described by Grynkiewicz. For this, HUVEC were exposed to 5 μM ionomycin in modified Tyrode’s buffer containing either 3 mM Ca²⁺ or 5 mM EGTA to obtain the maximum (R_max) and minimum (R_min) of the ratio of fluorescence (R), respectively. Afterwards, [Ca²⁺]i was calculated according to the equation: [Ca²⁺]i = K_d x Q x (R - R_min)/(R_max - R). K_d is the dissociation constant of fura 2 (10) and Q is the ratio of the 380-nm excitation signals of cells treated with ionomycin in presence of 5 mM EGTA and 3 mM Ca²⁺.

**Perfused rabbit lungs**: The model of perfused rabbit lungs has previously been described in detail (overview in¹¹). Briefly, rabbits of either sex were anticoagulated with heparin and
deeply anesthetized with ketamine/xyazine. Animals were tracheotomized and ventilated with room air using a Harvard respirator (Hugo Sachs Elektronik, Germany) (tidal volume, 30 ml; frequency, 30/min; positive end-expiratory pressure, 1 mm\(^3\) H\(_2\)O). Midsternal thoracotomy was performed, the pulmonary artery and the left atrium were cannulated, and perfusion with Krebs-Henseleit-buffer (pH 7.35-7.40) was started. After extensive rinsing of the lung vasculature, lungs were perfused in a recirculating system with a pulsatile flow of 100 ml/min (total volume, 150 ml); left atrial pressure was set at 2 mm Hg (referenced at the hilus). In parallel with the onset of artificial perfusion, ventilation was switched to a gas mixture containing 5% CO\(_2\), 21% O\(_2\), rest N\(_2\). Lungs were suspended from a force transducer for weight monitoring, and the whole system was equilibrated at 37°C.

K\(\text{fc}\) and total vascular compliance were determined gravimetrically from the slope of the lung weight-gain curve induced by a 7.5-mm Hg-step elevation of the venous pressure for 8 min. Lung weight gain was calculated as the difference in organ weight measured directly before and 5 min after each of these pressure-elevation maneuvers.\(^{11-13}\) Pulmonary arterial and venous pressures were monitored by pressure transducers. The microvascular pressure was determined by the arterial and venous double occlusion technique.

After an initial 30 min steady-state period with performance of a control hydrostatic challenge, time was set at \(t = -30\). Lungs were perfused in the presence or absence of \(10^{-7}\) mol/L ADM, followed by infusion of H\(_2\)O\(_2\) at \(t = -15\) min (100 \(\mu\)mol/min over 15 min), as indicated. K\(\text{fc}\)- and microvascular pressure measurements were performed at \(t = 30\) min, or 60 min. In all experiments, the thromboxane receptor antagonist BM 13.505 (5 \(\mu\)mol/L) was admixed to the recirculating buffer fluid at \(t = -10\) min prior to H\(_2\)O\(_2\) application. Perfusate samples for determination of cAMP were taken after 30, 60, and 75 min after ADM application and processed for enzyme-linked immunosorbent assay (ELISA) (Biotrend, Cologne, Germany) analysis.
**Statistical methods:** Depending on the number of groups (A) and the number of different time points studied (B) data of Fig. 1, 2, 6, and online Fig. 3 were analyzed by an A X B analysis of variance (ANOVA). Main effects were then compared by a F-probability test. A one-way ANOVA was used for data of Fig. 3, Fig. 4A, Fig. 6 and online Fig. 3 and followed by a post hoc Student-Newmann-Keuls test. If necessary, values were log transformed to achieve a normal distribution before statistical analysis. P < 0.05 was considered significant. Graphically data are displayed as mean ± SEM.
RESULTS:

Specific binding of human adrenomedullin to endothelial cells.

Competitive receptor binding studies were performed to assess specific binding of ADM to endothelial cells. Human $^{125}$I ADM binds to confluent HUVEC monolayers with a $B_{\text{max}}$ of 0.4 pmol/well and displayed a $K_d$ of about 147.9 nmol/L (Online Fig. 1A, 1B). Specificity of ADM binding to HUVEC was confirmed by competition experiments using increasing concentrations of unlabeled ADM (Online Fig. 1C). Confluent PAEC cultures showed a $B_{\text{max}}$ of 0.25 pmol/well and a $K_d$ of 13.4 nmol/L for human $^{125}$I adrenomedullin which could be blocked by increasing amounts of unlabeled ADM indicating specificity of peptide binding (data not shown).

ADM displayed no effect on $H_2O_2$-, thrombin- or HlyA-induced increase of $[Ca^{2+}]_i$ in endothelial cells.

ADM-exposure of HUVEC (1-100 $\mu$mol/L) had no effect on basal $[Ca^{2+}]_i$ content within a observation period of 15 min (data not shown). We next analyzed the effect of ADM-preincubation (10 $\mu$mol/L for 15 min) on thrombin-, $H_2O_2$- or HlyA-induced increase of endothelial $[Ca^{2+}]_i$ (Online Fig. 2). ADM pretreatment showed no effect on $H_2O_2$- (1 mmol/L) (Online Fig. 2A, B), thrombin- (0.1 U) (Online Fig. 2C, D), or HlyA- (0.1 HU) (Online Fig. 2E, F) related rise of $[Ca^{2+}]_i$.

ADM and/or $H_2O_2$-treatment displayed no substantial changes in mean pulmonary artery (mPAP) or mean pulmonary microvascular pressure (mMVP) in isolated rabbit lungs.

Since ADM is known to participate in the regulation of vascular tone which influences filtration pressure and thereby modulating fluid filtration, we monitored pulmonary artery as well
as pulmonary microvascular pressure in the isolated rabbit lungs (Online Fig 3A, B). In all experiments, the thromboxane receptor antagonist BM 13.505 (5 μmol/L) was admixed to the recirculating buffer fluid 10 min prior to H₂O₂ application to block H₂O₂-induced (thromboxane-mediated) pulmonary vasoconstriction. Lungs were exposed to H₂O₂ (100 μmol/min admixed to the 150 ml perfusate over 15 min) alone or pretreated with 0.1 μmol/L ADM 15 min before H₂O₂-admixture. Neither ADM-treatment alone nor administration of ADM and H₂O₂ in combination showed a significant effect on mMVP (Online Fig. 3B). mPAP in ADM and H₂O₂-treated lungs differs slightly at one time point (p = 0.046, t=15 min) from H₂O₂-exposed lungs (Online Fig. 3A). There were, however, no significant changes at the time points of the assessment, suggesting that the observed differences in vascular pressure did not underlie the differences in fluid filtration.
FIGURE LEGEND

Online Figure 1
Saturation binding curve of $^{125}$I adrenomedullin to HUVEC grown in 24 well plates. $K_d$: 147.9 nmol/L, $B_{\text{max}}$ of 0.4 pmol/well (Online Fig. 1A). $K_d$ and $B_{\text{max}}$ values were calculated from original data, not from Scatchard plot. Online Fig. 1B shows the linearization of the data according to Scatchard. Competition of unlabeled peptide and $^{125}$I ADM binding to HUVEC (Online Fig. 1C). Cells were incubated with 0.01 nmol/L $^{125}$I ADM in the presence of increasing concentrations of unlabeled peptide for 90 min at room temperature. Data presented in Online Fig. 1A and 1B are the average from triplicate determinations in a representative experiment. Data shown in Online Fig. 1C are means ± SEM of 3 separate experiments.

Online Figure 2
Effect of ADM-preincubation (10 µmol/L for 15 min) on thrombin-, $H_2O_2$- or HlyA-induced increase of endothelial [Ca$^{2+}$]i. ADM pretreatment showed no effect on $H_2O_2$- (1 mmol/L) (Online Fig. 2A, B), thrombin- (0.1 U) (Online Fig. 2C, D), or HlyA- (0.1 HU) (Online Fig. 2E, F) related rise of [Ca$^{2+}$]i. Representative tracings (out of three separate experiments) are shown.

Online Figure 3
Effect of ADM treatment on mean pulmonary artery (mPAP) and pulmonary microvascular pressure (mMVP) in the perfused isolated rabbit lungs. Lungs were exposed to 10 mmol/L $H_2O_2$ alone or were pretreated with 0.1 µmol/L ADM 15 min prior to $H_2O_2$-admixture. Neither ADM-treatment alone nor administration of ADM and $H_2O_2$ in combination showed a significant effect on mMVP (Online Fig. 3B). However, mPAP in ADM and $H_2O_2$-treated lungs dif-
fered significantly (# P = 0.046) at time point t=15 min from H₂O₂-exposed lungs (Online Fig. 3A). Data presented are mean ± SEM of five separate experiments.
Online Fig. 2
Online Fig. 1
Online Fig. 3
REFERENCES


