Endothelium

Aldosterone Modulates Endothelial Permeability and Endothelial Nitric Oxide Synthase Activity by Rearrangement of the Actin Cytoskeleton

Torsten Kirsch, Michaela Beese, Kristin Wyss, Uwe Klinge, Hermann Haller, Marion Haubitz, Anette Fiebeler

Abstract—Aldosterone (Aldo) is involved in vascular remodeling and inflammation; however, the mechanisms are imperfectly defined. We hypothesized that Aldo alters endothelial integrity and modifies paracellular permeability. Human umbilical vein endothelial cells were exposed to Aldo (10^{-9} mol/L) and alterations in paracellular permeability, assembly of tight and adherens junctions and activation of intracellular signaling pathways were determined. Aldo increased endothelial permeability for molecules ≤70 kDa within 60 minutes. A transient loss of cortical actin with formation of actin stress fibers and disruption of continuous adherens and tight junction strands accompanied these changes. Mineralocorticoid receptor blockade, inhibition of RhoA, or disruption of extracellular-regulated protein kinase 1/2 signaling pathways attenuated the Aldo-related effects. Moreover, Aldo-induced cytoskeletal rearrangement led to rapid dephosphorylation of protein kinase B and subsequent deactivation of endothelial nitric oxide synthase. In vivo tracer flux experiments with Evans blue–conjugated albumin demonstrated a concordant response to Aldo in freshly isolated umbilical arteries. Furthermore, low-dose cortisol (3×10^{-10} to 3×10^{-9} mol/L) mimics the effect of Aldo on endothelial integrity, and Aldo, by upregulating 11β-hydroxysteroid dehydrogenase type 2, might even aggravate this deleterious effect of low-dose cortisol. We suggest that these mechanisms may contribute to the vasculopathy induced by inappropriate mineralocorticoid receptor activation. (Hypertension. 2013;61:501-508.)

Key words: aldosterone ■ endothelial cells ■ vascular permeability ■ intercellular junctions

Endothelial cells form a thin monolayer lining the inner surface of blood and lymphatic vessels. The cells are connected by intercellular junctions, which control a variety of cellular properties, including adhesion, cell migration, paracellular transport, and leukocyte infiltration. In contrast to epithelial cells with their highly conserved junction structures, endothelial cells showed a higher variability with formation of adherens, tight and gap junctions along the entire cell–cell contact area. Moreover, the composition of endothelial tight and adherens junctions differs from that of epithelial cells. Thus, endothelial adherens junctions are mainly composed of vascular endothelial cadherin (VE-cadherin) and catenins linking the junctions to the actin cytoskeleton.

The main constituents of endothelial tight junctions are occludin, claudin-5, and the junctional adhesion molecules. These membrane-spanning proteins are anchored to the cytoskeleton via adaptor proteins, including zonula occludens (ZO) proteins, afadin, or cingulin. However, the cytoskeleton of the cell not only serves as an attachment point but also actively controls maintenance and assembly of tight and adherens junctions. In a variety of pathophysiological conditions, disassembly of endothelial tight junctions has been described, for example, ischemia-induced cerebral edema formation, viral or bacterial penetration, and cerebral edema attributed to malignant brain tumors. Several molecules are known to modulate the integrity of tight junctions. Although vascular endothelial growth factor, histamine, thrombin, or reactive oxygen species promote breakdown of endothelial tight junctions, transforming growth factor-β or synthetic steroids, such as dexamethasone (DM), protect junction architecture.6-9

Aldosterone (Aldo) regulates volume homeostasis in the kidney. In addition, Aldo has been shown to modify many pathophysiological processes, including inflammation, fibrosis, immune responses, and vascular integrity.10 Although hyperaldosteronism impairs vascular function and endothelial remodeling,11 mineralocorticoid receptor (MR) antagonists like spironolactone or eplerenone (EPL) show beneficial influences on vascular integrity and reduced cardiovascular mortality.12-13 The aim of the current study was to characterize the effect of Aldo on cytoskeletal rearrangement processes and on barrier properties in human endothelial cells.
Methods

An expanded Materials and Methods section can be found in the online-only Data Supplement.

Results

Aldo Modulates Paracellular Permeability In Vitro and Ex Vivo

We measured Aldo-induced alterations in vascular permeability toward Evans blue–conjugated albumin in human umbilical arteries. Preliminary experiments revealed that Aldo exerts its most prominent effect on paracellular permeability after 60 minutes at concentrations of $10^{-9}$ to $10^{-10}$ mol/L (Figure S1, available in the online-only Data Supplement). Therefore, the following experiments were performed with an Aldo concentration of $10^{-9}$ mol/L. After 60 minutes, albumin flux significantly increased compared with Ringer solution alone ($242\pm108 \mu g$ of BSA/mg of dry tissue versus $120\pm63 \mu g$ of BSA/mg of dry tissue; $P<0.001$). Elevated albumin extravasation could be prevented by pretreating the arteries with EPL for 30 minutes ($111\pm51 \mu g$ of BSA/mg of dry tissue; $P<0.001$; Figure 1A).

We then determined the effects of Aldo on paracellular permeability in cAMP-treated human umbilical vein endothelial cells (HUVECs). To test whether the density of endothelial monolayer influenced expression of the MR, transcript amounts were analyzed by real-time quantitative PCR. MR mRNA level decreased with increasing density of the HUVEC monolayer, and stimulation with cAMP even further reduced the amount of MR transcript (Figure 1B). However, intracellular cAMP levels were not regulated by Aldo itself ($10^{-6}$ to $10^{-11}$ mol/L; data not shown). Tracer flux assays revealed that Aldo induced a clear, time-dependent transient increase in paracellular permeability toward both 10 and 70 kDa dextrans with a peak observed after 60 minutes ($7.9\pm1.6$-fold increase for the 10 kDa dextran and $1.8\pm0.3$-fold increase for the 70 kDa dextran; $P<0.01$; Figure 1C). In HUVECs that were not treated with cAMP, Aldo induced a much lower but still significant increase in permeability toward the 10-kDa dextran, whereas paracellular flux of the 70-kDa dextran did not change at any time point (Figure 1D).

Aldo Induces Remodeling of the Actin Cytoskeleton and Alters Assembly of Adherens and Tight Junctions

Next, we analyzed Aldo-dependent changes in actin distribution in cAMP-treated human umbilical vein endothelial cells (HUVECs). To test whether the density of endothelial monolayer influenced expression of the MR, transcript amounts were analyzed by real-time quantitative PCR. MR mRNA level decreased with increasing density of the HUVEC monolayer, and stimulation with cAMP even further reduced the amount of MR transcript (Figure 1B). However, intracellular cAMP levels were not regulated by Aldo itself ($10^{-6}$ to $10^{-11}$ mol/L; data not shown). Tracer flux assays revealed that Aldo induced a clear, time-dependent transient increase in paracellular permeability toward both 10 and 70 kDa dextrans with a peak observed after 60 minutes ($7.9\pm1.6$-fold increase for the 10 kDa dextran and $1.8\pm0.3$-fold increase for the 70 kDa dextran; $P<0.01$; Figure 1C). In HUVECs that were not treated with cAMP, Aldo induced a much lower but still significant increase in permeability toward the 10-kDa dextran, whereas paracellular flux of the 70-kDa dextran did not change at any time point (Figure 1D).

![Figure 1](http://hyper.ahajournals.org/)

Figure 1. Aldosterone (Aldo) alters permeability in umbilical arteries and in human umbilical vein endothelial cells (HUVECs). **A**, Aldo increased extravasation of Evans blue–conjugated albumin in umbilical arteries, whereas pretreatment with EPL circumvented this effect. **B**, Endothelial mineralocorticoid receptor (MR) transcript levels were influenced by cell confluence and density. **C** and **D**, Aldo increased paracellular flux of fluorescein isothiocyanate (FITC)-dextrans in HUVECs pretreated with or without pCPT-cAMP. EPL indicates eplerenone. **P<0.001; *P<0.05.**
**Figure 2.** Aldosterone (Aldo) induces formation of F-actin stress fibers and disrupts junction strands. **A**, Aldo induced disassembly of cortical actin, generation of actin stress fibers, and disruption of continuous cell–cell contacts in human umbilical vein endothelial cells. Shown are stainings for F-actin, vascular endothelial (VE)-cadherin, zonula occludens 1 (ZO-1), occludin, and claudin-5. **B**, Also in non-cAMP–treated cells Aldo induced disassembly of cortical actin and cell–cell junctions. **C**, Repeated exposure to Aldo exerts similar effects on VE-cadherin, ZO-1, and claudin-5 distribution. **D**, Aldo induced increased mRNA expression of ZO-1 and claudin-5. *P<0.05. Scale bar represents 25 μm.
HUVECs cultivated without cAMP, rearrangement of cortical actin structures and reassembly of continuous junction strands were less prominent after 4 hours, suggesting that exposure to external cAMP did not alter the effect of Aldo on cell contraction and junction disassembly but induced a much faster resealing of intercellular contacts in endothelial cells (Figure 2B). Moreover, re-exposure to Aldo for 60 minutes showed comparable effects on junction disassembly compared with a single exposure, demonstrating that the response to Aldo is not sensitive to tachyphylaxis (Figure 2C). Stimulation with Aldo for ≤4 hours had no significant effect on mRNA expression of VE-cadherin and occludin but induced a slight increase in claudin-5 and ZO-1 expression (Figure 2D).

**Inhibition of the MR Restored Junctional Integrity**

To elucidate involvement of the MR in Aldo-induced alterations of the cytoskeleton and junction architecture, HUVECs were pretreated with the MR inhibitor EPL (10⁻⁵ mol/L) before exposure to Aldo. EPL partially prevented the formation of F-actin stress fibers compared with cells that were vehicle treated. Aldo-induced disruption of continuous junction strands could also be attenuated by EPL (Figure 3A). Moreover, pretreatment with EPL significantly reduced permeability in Aldo-treated cells (Figure 3B).

**Effect of Aldo Is Mediated by Extracellular-Regulated Protein Kinase1/2 and RhoA**

Exposure to Aldo induced a significant phosphorylation of the extracellular signal-regulated kinase (ERK) 1/2. Concordantly, blocking ERK1/2 phosphorylation successfully prevented disassembly of cortical actin rings and continuous junction strands in response to Aldo (Figure 4A). Because formation of actin stress fibers suggests involvement of the RhoA/ROCK pathway, we performed RhoA activation assays. Aldo induced a very rapid activation of RhoA within the first minutes, and inhibition of the RhoA/ROCK pathway prevented formation of actin stress fibers in response to Aldo (Figure 4B).

**Aldo Regulates Activity of Protein Kinase B and Endothelial NO Synthase**

Next, we analyzed the time course of protein kinase B (AKT) and endothelial nitric oxide (NO) synthase (eNOS) phosphorylation in response to Aldo. Phosphorylation of AKT and of eNOS at Ser1177 increased enzyme activity, whereas phosphorylation of eNOS at position Tyr657 attenuated eNOS activation and NO generation. AKT was dephosphorylated 5 to 10 minutes after cells were exposed to Aldo and returned toward baseline after 30 to 45 minutes. The same temporal pattern could be observed for phosphorylation of eNOS at Tyr657, whereas dephosphorylation of eNOS at Ser1177 showed a peak after 15 minutes (Figure 5A). Concordantly, nitrate/nitrite level decreased 10 to 15 minutes after addition of Aldo, whereas pretreatment with EPL partly prevented the decline in NO production (Figure 5B). Dephosphorylation of AKT and of (Ser1177) eNOS, as well as decreased NO production in response to Aldo, could be circumvented by inhibition of the RhoA/ROCK pathway (Figure 5C), whereas constitutively active AKT prevented neither Aldo-induced permeability nor cytoskeletal or cell junction rearrangement (data not shown).

**Cortisol Partly Mimics the Effect of Aldo on Endothelial Integrity**

Because the MR is also targeted by glucocorticoids, we asked whether cortisol could mimic or counteract the effect of Aldo in endothelial cells. We, therefore, measured paracellular permeability toward 70 kDa dextran in response to increasing concentrations of cortisol. As shown in Figure 6A, low levels (3×10⁻¹⁰ to 3×10⁻⁹ mol/L) of cortisol significantly increased paracellular permeability and influenced distribution of junction-associated proteins as shown by immunostaining for ZO-1. Cortisol is converted to the inactive cortisone by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). Exposure to Aldo induced a significant increase in endothelial 11βHSD2 both on transcript and on protein level (Figure 6B), which possibly aggravates the deleterious effect of cortisol on endothelial function. On the other side, exposure to cortisol led to an only moderate increase in ERK activation (Figure S2). Also, nitrate/nitrite release was not influenced by cortisol (data not shown).

**Discussion**

Primary and secondary hyperaldosteronism are receiving increasing attention because Aldo affects multiple cellular responses and modulates vascular and tissue remodeling cascades. Here, we demonstrated that Aldo temporarily increases vascular permeability and modulates rearrangement of the actin cytoskeleton and tight and adherens junctions. These responses were accompanied by a RhoA/ROCK-dependent decrease of AKT and eNOS activity.
Cell–cell junctions control a variety of crucial processes, such as cell adhesion, polarity, permeability, or leukocyte transmigration, and disassembly of cell–cell junctions is linked to several pathological conditions. Two major groups of hormones of the adrenal gland belong to the physiological mediators that alter junctional architecture, glucocorticoids and mineralocorticoids. Glucocorticoids like cortisol or the synthetic derivates DM and triamcinolone acetonide have been shown to induce formation of tight junctions in both epithelial and endothelial cells in vitro. Moreover, because of their junction-stabilizing effects, glucocorticoids successfully counteract breakdown of tight junction structures under inflammatory conditions like oxidative stress or enhanced tumor necrosis factor-α exposure. However, most of those studies analyzed endothelial junctions or barrier function in response to DM or triamcinolone acetonide, while in contrast to cortisol, these synthetic glucocorticoids have only minor, if any, effect on the MR. In this study, we could demonstrate that exposure to low-dose cortisol mimics the effect of Aldo on the endothelium rather than counteracting it. This finding might be explained by the affinity of cortisol to the MR at physiological levels. Moreover, most of the studies analyzing glucocorticoids chose longer exposure periods (>24 hours), and rather few data exist on short-term effects of cortisol on endothelial barrier properties. In this regard, it is noteworthy that Aldo induced the expression of 11βHSD2 in a very rapid and thus nongenomic way, leading to an even lower cortisol level. Hence, our results strengthen the hypothesis that MR activation reflects not necessarily Aldo-mediated effects but also those of glucocorticoids.

Aldo, the most important mineralocorticoid, has mainly been studied for its influence on tight junctions in the kidney. In renal collecting duct cells, Le Moellic et al found that Aldo (10−9 mol/L) rapidly changed permeability by modulating phosphorylation of claudin-4. Other investigators demonstrated that, in the colon, Aldo mediates alterations in Na+ absorption, paracellular permeability, and resistance. In vascular cells, Aldo leads to RhoA-dependent formation of actin stress fibers, cytoskeletal remodeling, and cell migration. Others showed that Aldo induces augmented release of Weibel-Palade bodies and triggers enhanced adhesion of leukocytes. Both effects may be explained by the modifying effect of Aldo on tight junctions as delineated in this study.

Moreover, disorganized cell–cell junctions are considered to be a prerequisite for tumor-induced angiogenesis, and the...
recent findings that Aldo is capable of modulating expression of proteins associated with angiogenesis, neovascularization, or vascular leakage are of particular interest and support the hypothesis of Aldo being a promoter of vascular rearrangement and (neo-)angiogenesis.33–35

In this study, we observed not only cytoskeletal and tight junction rearrangement processes in response to Aldo but also changes in the distribution pattern of VE-cadherin. VE-cadherin–containing adherens junctions have been described to actively control proper tight junction formation, and the question as to what extent Aldo-induced VE-cadherin redistribution actively influences the composition and integrity of tight junctions remains to be determined.36 On the other hand, Aldo-induced actin stress fiber formation and subsequent cell contraction might be the initial cause for altered junction distribution.

Our findings, that RhoA and ERK1/2 inhibition prevented cytoskeletal and junction rearrangement, demonstrate that, at least in part, Aldo modulates actin rearrangement and endothelial barrier function via the RhoA/ROCK pathway. Moreover, our results regarding enhanced expression of junction proteins in response to Aldo are in line with other studies showing that Aldo, via genomic and nongenomic mechanisms, modulates expression of several genes in endothelial cells.31,37–39

Notably, there was a strong temporal correlation among junction rearrangement, enhanced permeability, and deactivation of AKT and eNOS. Basile et al40 have demonstrated that plexin-B1–induced activation of RhoA/ROCK leads to enhanced activation of Pyk2, which, in turn, activates phosphatidylinositol 3-kinase, AKT, and ERK. In addition to activation of the phosphatidylinositol 3-kinase/AKT pathway, Pyk2 is known to be responsible for phosphorylation of eNOS at Tyr657, which leads to attenuation of enzyme activity. Others have shown that increased RhoA activity modulates phosphorylation of both the mitogen-activated protein kinase and the phosphatidylinositol 3-kinase/AKT pathway.41,42 A recently published study showed that Aldo decreases endothelial NO levels and modulates pulmonary arterial hypertension.43 Our results, in combination with these findings, suggest a model in which mineralocorticoids like Aldo modulate endothelial integrity by MR-dependent redistribution of cytoskeletal and junction structures, enhanced paracellular permeability, and altered eNOS activity.

Perspectives

We have shown that Aldo is capable of inducing rearrangement processes of the endothelial actin cytoskeleton leading
to alterations of cell–cell junctions, barrier properties, and NO production. We also introduced the first data on low-dose cortisol effects on endothelial integrity. These findings pave the way for further studies on corticoid-mediated vascular alterations in vitro, as well as in experimental animal models addressing target-organ damage.

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Disclosures
None.

References


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Aldosterone modulates endothelial permeability and eNOS activity by rearrangement of the actin cytoskeleton

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Running title: Aldosterone modulates vascular permeability

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Detailed Methods

Chemicals
If not stated otherwise, all chemicals were obtained from Sigma (Sigma, Aldrich, Munich, Germany). EPL was diluted in DMSO (Stock solution was 10^{-3} mol/L) and Cortisol was diluted in Ethanol (Stock solution was 10^{-3} mol/L). For all assays appropriate vehicle controls were conducted.

Cell culture
Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords as described earlier. Cells were cultivated in EGM-2 medium (Lonza, Vervier, Belgium). The Hannover Medical School Ethics Committee approved the study and written informed consent was obtained from all human participants in accordance with the Declaration of Helsinki.

Ex-vivo permeability assay
Umbilical arteries were carefully dissected from the umbilical cord and washed with 20 ml cold PBS containing Ca^{2+} and Mg^{2+} (Lonza). Evans blue (EB)-conjugated albumin (EBA) was prepared by diluting a 2% EB stock solution in 4% bovine serum albumin/Ringer's solution (BSA) to a final concentration of 0.33%. Arteries were perfused with EBA solution or with EBA solution containing 10^{-9} mol/L Aldo for 60 min. In some experiments arteries were exposed to EPL (10^{-5} mol/L) prior to perfusion with Ald. Arteries were intensively washed with PBS to remove any residual EBA, and incubated with formamide for 24 h at 58°C. Absorbance of EB was measured at 595 nm and referenced to an EBA standard curve. Vascular flux of albumin was expressed as µg BSA/mg dry tissue.

Paracellular tracer flux assay
HUVEC were seeded into the upper compartment of transwell devices with a pore size of 0.4 µm. For some experiments cells were stimulated with 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (pCPT-cAMP, Sigma-Aldrich, Munich, Germany) for 72 h. Fluorescein (FITC)-coupled dextrans (FD) with different molecular masses were added to the upper compartment 15 minutes prior to the indicated time points. Fluorescence of the dextrans in the lower compartment was measured by an ELISA reader equipped for fluorescence measurement (Tecan, Crailsheim, Germany) and concentration was determined according to a FITC-dextran standard curve.

Transfection
The plasmid coding for a constitutive active AKT was a gift from Richard Roth (Addgene plasmid #10841). HUVEC were transfected the day after seeding using XtremeGene transfection reagent (Roche Applied Science). 24 hours after transfection the cells were used for the appropriate assays.
**Nitrate/Nitrite Analysis**
For measurement of nitrate/nitrite release cells were stimulated for the indicated time periods, the supernatant was collected, centrifuged and nitrate/nitrite level were determined using the Nitrate/Nitrite Colorimetric Assay from Cayman Chemicals (Ann Arbor, MI) according to the manufacturer’s protocol.

**RNA extraction and real-time quantitative RT-PCR**
Total RNA was extracted on RNeasy mini columns (Qiagen, Hilden, Germany) and 2 µg of total RNA was reverse transcribed with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany). Real-time qPCR was carried out on a LightCycler480-II System (Roche) using FastStart Polymerase (Roche) and SYBR-Green I (Invitrogen, Karlsruhe, Germany). Gene-specific oligonucleotides for Occludin, Claudin-5, MR, ZO-1 and VE-cadherin were obtained from Qiagen (QuantiTect Primer Assay) with the following corresponding ordering numbers: Occludin (QT00081844); Claudin-5 (QT0168123); MR (QT00028490); ZO-1 (QT00077308); VE-cadherin (QT00013244). PCR results were normalized to the expression of ribosomal protein 13A (RPL13A). The sequences of the RPL13A oligonucleotides were 5´-GTGTTTGACGCCATCCCATCC-3´ and 5´-CTTCAGACGCACGACCTTTGA-3´.

**Western blot**
Cells were lysed in ice-cold 1 x lysis buffer (NEBiolabs, Frankfurt, Germany) containing protease and phosphatase inhibitor cocktail tablets (Roche) and protein amount was determined with a BCA protein assay kit (Thermo Fisher Scientific, Bonn, Germany). Cell lysates (50 µg) were separated by SDS PAGE electrophoresis and blotted on to a PVDF nylon membrane. Membranes were incubated with the appropriate primary antibody followed by incubation with a horseradish peroxidase- (HRP-) conjugated secondary antibody (Cell Signaling/NEBiolabs). Bands were visualized by Western Lighting chemiluminiscence reagent (Perkin Elmer, Rodgau, Germany) and quantified by densitometry using a CCD camera and Quantity One software (Biorad Laboratories, Munich, Germany). Primary antibodies used were anti-p-ERK1/2, anti p-AKT, anti-ERK1/2, anti-AKT (Cell Signaling, Danvers, MA), anti-eNOS, anti-pSer1177-eNOS (Biotrend, Cologne, Germany) and anti-pTyr657-eNOS (ECM Biosciences, Versailles, KY). Equal protein loading was verified by stripping off the original antibodies and reprobing the membrane with a mouse anti-beta-tubulin antibody (BD Pharmingen, Heidelberg, Germany).

**RhoA activity**
RhoA activity was determined in a RhoA activation assay according to the manufacturer’s instructions (Cytoskeleton, Denver, Co). In brief, cells were exposed to Ald (10^-9 mol/L) for up to 15 min and lysates were snap-frozen in liquid N2. Active RhoA was pulled-down on Rhotekin-coupled beads and quantified by western blotting using an anti-RhoA antibody. Total RhoA and beta-tubulin was used for normalization of data.

**Immunocytochemistry**
Cells were grown on collagen-coated glass cover slips and fixed in ice-cold acetone for 15 min at -20°C followed by permeabilization with ice-cold methanol for 20 min at -20°C. After washing with PBS cells were blocked in donkey serum, incubated with the primary antibody for one hour followed by incubation with the appropriate secondary antibody
coupled to ALEXA-488 or ALEXA-546 (Invitrogen) for an additional hour. DNA was counterstained with DAPI (Sigma Aldrich). Confocal images were taken on a Leica DM IRB microscope with a TCS SP3 AOBS scan head equipped with argon and krypton laser beams and a 405 nm laser. Micrographs were obtained with a HCX PL APO 63x1.4 numerical aperture objective. Antibodies used in this study were a murine monoclonal anti-VE-Cadherin (clone 55-7H1 from BD Pharmingen), anti-ZO-1 antibody (clone 1/ZO-1 from BD Pharmingen), anti-Occludin antibody (clone 3F10 from Invitrogen), a polyclonal anti-11BHSD2 antibody (Cayman Chemical) and a rabbit anti-Claudin-5 antibody (Santa Cruz Biotechniques, Heidelberg). For visualizing F-actin cells were fixed with 4% paraformaldehyde for 15 min at 4°C, permeabilized with 0.1% Triton-X100 and stained with phalloidin coupled to Alexa-546 (Invitrogen).

**Statistical analysis**
For comparison of more than two groups of individuals, the nonparametric Kruskal-Wallis test was used. If significant differences in between the groups were found the Mann-Whitney U test was used to calculate the difference between each pair of groups. For the in vitro data Student’s T test or Mann-Whitney U test was applied depending on the distribution of the data. p-values (2-sided) were considered significant at p<0.05. Statistical analysis was performed using SPSS Statistics 19 (SPSS, Muenchen, Germany).
**S1:** Time and dose responses of Aldo on paracellular permeability towards 10kDa and 70 kDa dextrans. (A) HUVEC were treated with different concentrations of Aldo and paracellular permeability was measured for a time period of two hours. (B) Time response of Aldo at a concentration of $10^{-9}$ mol/L was determined. For these assays FD10 and FD70 were added to the upper compartment of transwell devices 15 minutes before measurement.

**S2:** Cortisol-induced phosphorylation of pERK1/2 in HUVEC. HUVEC were exposed to Cortisol ($3 \times 10^{-7}$ mol/L) for different time points, fixed and stained with a phospho-specific anti-ERK antibody. Nucleus was counterstained with Dapi. scale bar represents 25 µm.