Research Square

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Modulation of the Permeability-Inducing Factor Angiopoietin-2 Through Bifonazole in Systemic Inflammation

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Abstract

Background

Sepsis is a life-threatening organ dysfunction due to a pathological host response to an infection. Vascular barrier breakdown represents a key component of the maladaptive host response and the release of pre-stored endothelial Angiopoietin-2 (Angpt-2) is a direct driver of endothelial hyperpermeability. Although it has been demonstrated that Angpt-2 is associated with morbidity and mortality, a therapeutic approach targeting this injurious endothelial protein is not available. We screened for FDA approved drugs that might have off-target effects decreasing circulating Angpt-2 levels and therefore, ameliorating capillary leakage.

Methods

Endothelial cells were isolated from human umbilical veins (HUVECs) and used for *in vitro* studies at baseline and after stimulation (FDA-library screening, RT-PCR, ELISA, immunocytochemistry). On the functional level, we assessed real-time transendothelial electrical resistance (TER) using the ECIS (electric cell-substrate impedance sensing) device.

Results

We identified Bifonazole (BIFO) in an unbiased library screen and found that it is able to reduce spontaneous Angpt-2 release in HUVECs in a time- and dose-dependent manner after 8, 12 and 24 h (24 h: veh: 15.6 ± 0.7 vs. BIFO: 8.6 ± 0.8 ng/mL, $p < 0.0001$). BIFO reduced not only Angpt-2 release but also its intra-cellular content by 33 % ($p < 0.001$). Stimulation of HUVECs with a sepsis mediator, i.e. tumor necrosis factor α (TNFα) (10 ng/ml) induced a rapid release of Angpt-2 that could analogously be blocked by additional treatment with BIFO (veh: 1.58 ± 0.2 vs. BIFO: 1.02 ± 0.1, $p < 0.0001$). On the functional level, the quantification of endothelial permeability by TER revealed that BIFO was sufficient to reduce an acute barrier breakdown induced by Thrombin (veh: 0.82 ± 0.1 vs. BIFO: 1.01 ± 0.02, $p < 0.05$).

Conclusion

The antifungal BIFO can reduce both release and biosynthesis of the endothelial-destabilizing factor Angpt-2 *in vitro*. Henceforth, our results suggest that BIFO might counterpart the pathophysiology of endothelial permeability in systemic inflammation. Additional studies are needed to further investigate the underlying mechanism and to translate these findings to *in vivo* models.

Background:

The definition of sepsis was updated to the so-called “sepsis-3 definition” in 2016 [1]. Sepsis is now defined as a dysregulated host response to infection with life-threatening organ dysfunction [1–3]. If residual organ perfusion cannot supply the cellular oxygen demand, it can progress to septic shock and ultimately, death [1, 2]. In 2017, 48.9 million sepsis cases were recorded worldwide with striking 11.0

million people dying [4]. Sepsis-related deaths therefore, represent almost one fifth of all global deaths [4]. Its mortality is up to 55% for severe sepsis [5]. Recently, the World Health Organization (WHO) recognized sepsis as a global health priority [6]. Nevertheless, sepsis, septic shock and its often fatal outcome are not very well-known by the broad public in most countries [7].

The vast majority of sepsis research is currently focusing on highly complex humoral and immune pathways rather than vascular pathophysiology [8, 9]. However, in sepsis the endothelial dysfunction seems to play an central role in the pathological host response as virtually all physiological functions of the endothelium appear to be altered throughout the body [8, 10]. Breakdown of the endothelial barrier that clinically leads to the so-called *capillary leakage syndrome* is a key contributor of multiple organ failure [11, 12]. The Angiopoietin (Angpt)-Tie2 system is an important molecular regulator of these pathophysiological processes [9, 13, 14]. The transmembrane tyrosine kinase Tie2 is constitutively activated by its protective ligand Angiopoietin-1 (Angpt-1) in quiescence endothelium [9, 13, 14]. Angpt-1 ligation leads to Tie2 phosphorylation and activates canonical downstream pathways that maintain endothelial function and integrity [9, 13, 14]. Its antagonist Angiopoietin-2 (Angpt-2) is pre-stored within Weibel-Palade bodies of endothelial cells and can be released upon an injurious stimuli, e.g. systemic inflammation [14]. Tie2 deactivation by excess Angpt-2 leads to endothelial mediated inflammation and increases endothelial permeability [9, 15–17]. Of note, circulating levels of Angpt-2 in septic patients are closely associated with morbidity and mortality [15–17]. Therapeutic strategies that target Angpt-2 have been shown to be effective in animal models of sepsis [18, 19]. However, so far, no approved treatment targeting this endothelium-destabilizing system is available.

In order to overcome inheritant difficulties with drug development, we analyzed if approved drugs with well-known safety profiles might have beneficial off-target effects on the release of Angpt-2 in endothelial cells. We therefore screened an FDA-drug library and identified the antifungal Bifonazole (BIFO) as a potential negative modulator of Angpt-2 *in vitro*. We hypothesized that BIFO might decrease endothelial Angpt-2 release thereby improving vascular barrier function.

Results:

Identification of Bifonazole as a possible Angiopoietin-2 (Angpt-2) suppressor

By screening a drug library for FDA-approved drugs that might affect the release of Angpt-2 from endothelial cells, we identified different potential suppressors of Angpt-2. Here we investigated the anti-fungal Bifonazole (BIFO) that was able to suppress Angpt-2 up to ten times (Fig. 1) making it a suitable candidate for further investigation.

Bifonazole reduces baseline Angpt-2

In order to confirm the Angpt-2 suppressing effect of BIFO, we performed experiments on human umbilical vein endothelial cells (HUVECs). A dose course revealed that 10 µM BIFO was sufficient to

reduce the spontaneous release of Angpt-2 from HUVECs into the supernatant (Fig. 2A). A time course with 10 µM BIFO showed that Angpt-2 release in the supernatant was already reduced after 8 hours of treatment with the most prominent effect at the latest tested time-point (i.e. 24 hours) (veh: 15.6 ± 0.7 vs. BIFO: 8.6 ± 0.8 ng/mL, $p < 0.0001$) (Fig. 2B). Additionally, intracellular Angpt-2 – assessed by ELISA from protein lysate after 24 hours - was reduced in HUVECs treated with BIFO for 24 hours by 33% ($p < 0.0005$) (Fig. 2C). Fluorescent immunocytochemistry for Angpt-2 and von-Willebrand factor (vWF) confirmed these results. In BIFO treated HUVECs, Angpt-2 was significantly decreased, whereas vWF (that is stored within the same intracellular vesicles) was unchanged – indicating a specific inhibitory mechanism of BIFO on Angpt-2 (Fig. 2D). Given the observed reduction of Angpt-2 in the endothelial lysate, we hypothesized that Angpt-2 transcription might be affected by BIFO. However, this hypothesis could not be confirmed (**fig. S1A**). Together, BIFO reduces both Angpt-2 release and biosynthesis in a time- and dose-dependent manner but independently from its transcription.

Bifonazole reduces Angpt-2 after stimulation

As BIFO was able to reduce spontaneous Angpt-2 release and biosynthesis, we analyzed its effect on stimulated cells. Therefore, HUVECs were stimulated with three different mediators known to be involved in sepsis pathophysiology such as the cytokine *tumor necrosis factor α* (TNFα) [20]. TNFα was able to induce Angpt-2 release about 1.5 fold of its baseline level, whereas additional BIFO treatment significantly reduced Angpt-2 in the supernatants of these stimulated cells to a level known from unstimulated endothelial cells (veh: 1.6 ± 0.2 vs. BIFO: 1.0 ± 0.1 ng/mL, $p < 0.0001$) (**Fig. 3AB**). Although Angpt-2 could be decreased by BIFO treatment in the supernatants, mRNA transcription was again not involved in this regulation (**fig. S1B-C**). Phorbol-12-myristate-13-acetate (PMA) is a strong inductor of exocytosis of Weibel-Palade bodies [21]. Consistently, we observed an increase of Angpt-2 in the supernatants up to five times. Even under these extreme conditions, BIFO was still sufficient to reduce Angpt-2 levels in the supernatant by almost 50% after 12 hours and 24 hours ($p < 0.0001$) (**Fig. 3AB**). This decrease let to the hypothesis that BIFO might mechanistically inhibit the exocytosis of Angpt-2 from Weibel-Palade bodies. However, different studies with exocytosis inhibitors did not confirm this assumption (**fig. S2A-C**). Lastly, BIFO also reduced the Angpt-2 releasing effect of Thrombin (veh: 1.2 ± 0.04 vs. BIFO: 0.5 ± 0.06 ng/mL, $p < 0.0001$) (**Fig. 3AB**). Together, the data indicate that BIFO counteracts the induction of Angpt-2 release independently from the endothelium mediator.

Functional barrier improvement

As BIFO was able to reduce Angpt-2 levels in the supernatant in stimulated HUVECs, its effect was further tested in functional settings to precise predictions on its assumed protective effect on the endothelium. First, transendothelial electrical resistance (TER) was measured at different time points in order to objectively quantify endothelial permeability. As BIFO showed its strongest Angpt-2 inhibitory effect after 24 hours, HUVECs were incubated with BIFO for 24 hours and then stimulated with Thrombin. Thrombin temporarily induced capillary leakage, which lead to a decrease in impedance measured over cell membranes. BIFO partially protected this hyperpermeability shown by higher impendences throughout

the whole experiment (Fig. 4A). Greatest differences were shown after 2.5 hours (veh: 0.82 ± 0.10 vs. BIFO: 1.01 ± 0.02, $p < 0.05$) (Fig. 4B).

Additionally, a classical Transwell-Assay was performed to confirm BIFO's protective functional anti-permeability effect (Fig. 4C). Within the Transwell-Assay, hyperpermeability can be measured by the collected flow-through through an endothelial monolayer that directly correlates with the endothelial permeability. HUVECs were co-stimulated with BIFO and Thrombin and flow-through was analyzed. As expected, permeability was clearly increased upon Thrombin stimulation and BIFO could antagonize this effect significantly after 8, 12 (veh: 2.21 ± 0.51 vs. BIFO: 1.35 ± 0.23, $p < 0.001$) and 24 hours (Fig. 4C).

After quantifying BIFO's protective effect on the endothelium, endothelial cellular architecture was visualized. HUVECs were again treated with BIFO and Thrombin. Fluorescent immunocytochemistry was performed for VE-Cadherin, F-Actin and 4',6-diamidino-2-phenylindole (DAPI) (Fig. 4D). In healthy HUVECs structural proteins like F-Actin were ordered appropriately in a cortical configuration and intercellular junctional proteins, represented by VE-Cadherin, were clearly visible and continuously expressed surrounding every cell. In Thrombin treated HUVECs, however, the cytoskeleton was massively stressed and intercellular interaction disconnected indicating the morphological correlate of the former functional permeability experiments. To better visualize the locations where leakiness occurs most distinctively, we further schematically mapped the disconnected cells. Leakage was still visible in BIFO and Thrombin-treated cells; however, it was strongly reduced (Fig. 4D).

In summary, our observations indicate that BIFO can effectively counteract mediator induced endothelial permeability (most likely via Angpt-2 reduction).

Discussion:

Here we systematically screened an FDA drug library for potential candidates that might reduce endothelial Angpt-2 in a drug-repurposing context. Using this unbiased approach, the antifungal Bifonazole (BIFO) was found as a possible suppressor of Angpt-2. A series of *in vitro* experiments confirmed BIFO's off-target effect on Angpt-2 biosynthesis and exocytosis in a dose- and time dependent manner. Moreover, functional analysis demonstrated that BIFO was sufficient to ameliorate mediator induced endothelial permeability.

So far, neither this drug nor any other antifungal have been implicated in angiopoietin processing. First, we analyzed BIFO's effect on endothelial Angpt-2 release under baseline (i.e. unstimulated) and mediator-stimulated conditions. Given that sepsis-mediators drive the release of Angpt-2 into the supernatant, it is of no surprise that BIFO's effect was most potent in the context of this situation. We used as mediators both TNFα (an essential contributor to sepsis pathology in humans) and Thrombin (a strong inductor of vascular leak) both of which have been reported to be elevated during systemic inflammation in men [22, 23]. Additionally, PMA, that is known to be a strong inducer of Weibel-Palade-body exocytosis, the primary source of Angpt-2 [21, 24], was used as a third mediator. All three stimuli showed a strong and reproducible phenotype of vascular leakage. Most importantly, BIFO co-stimulation was sufficient to

reduce this pathologically driven Angpt-2 release into the supernatant in all three conditions. Besides this effect on Angpt-2 release, BIFO also reduced the intracellular Angpt-2 content indicating a potential additional effect on Angpt-2 biosynthesis.

Mechanistically, we aimed to differentiate if reduced Angpt-2 in the supernatant was a rather direct effect on exocytosis or an indirect effect via reduction of *de novo* protein synthesis. Indeed, the observed decrease in intracellular Angpt-2 suggested a role of protein synthesis although Angpt-2 transcription – as it has been earlier shown for the anti-migraine drug Flunarizine [20] – was unaffected upon BIFO stimulation (**fig. S1A-C**). As a potential posttranslational modification, we next analyzed degradation processes via the canonical ubiquitin-pathway that was also unaffected (data not shown). Finally, we analyzed BIFO's effect on exocytosis. PMA is a strong inducer of Weibel-Palade body and therefore, Angpt-2 exocytosis [21, 24]. As intracellular calcium plays an important role in exocytosis regulation, we analyzed the effects of the t-type calcium channel blockers Mibefradil and TTA-A2 [25, 26] (**fig. S2AB**). Additionally, we co-stimulated HUVECs with the nitric oxygen synthase (NOS) inhibitor N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-Name) [27] (**fig. S2C**). As BIFO's Angpt-2 lowering effect is not affected by any of these co-stimulations, neither intracellular calcium nor nitric oxygen concentration seem to be involved in BIFO's Angpt-2 regulating mechanism. Further experiments in order to detect the exact mechanisms are necessary.

We have also investigated the functional consequences on endothelial barrier function in the context of BIFO-driven Angpt-2 reduction. The barrier protective effect of an Angpt-2 lowering strategy is based upon an antagonizing effect on its membrane bound target receptor (i.e. Tie2) [9, 15–17]. We used well established *in vitro* models of endothelial hyperpermeability in response to Thrombin stimulation and assessed its effect in two different assays of permeability. TER was recorded as a highly sensitive measure of real-time permeability at a given moment, whereas a classical Transwell assay was used to detect the accumulation of a labeled macromolecule over an endothelial monolayer over time [28, 29]. In both assays BIFO co-stimulation of endothelial cells was sufficient to reduce spontaneous and mediator induced vascular barrier breakdown. Together, BIFO not only reduces Angpt-2, but also stabilizes the endothelial barrier function in an experimental setting *in vitro*.

BIFO is a substituted imidazole antifungal that is mainly used for topical application on local mycosis [30]. It has a broad activity against different fungal species and even some gram-positive bacteria [30]. BIFO canonically inhibits the ergosterol synthesis by inhibition of the cytochrome P450 depending 14- α demethylase and inhibition of HMG-CoA reductase which ultimately leads to malformation of fungal cell membrane [30–33]. As BIFO interferes with the HMG-CoA reductase [30, 33], a reduction of the transcription factor FOXO-1 might be involved according to Ghosh CC *et al.* [34]. However, the effect of BIFO on Angpt-2 does not seem to involve its mRNA transcription (**fig. S1A-C**). Therefore, no further transcription factors of Angpt-2 biosynthesis were examined.

Recently, anti-inflammatory effects of BIFO via decreasing intercellular adhesion molecule (ICAM)-1 expression have been shown in human skin model systems [32]. It has been shown that ICAM-1 is

regulated by Angpt-2 and is substantially increased during systemic inflammation [35, 36]. As Huth *et al.* showed a similar anti-inflammatory effect of BIFO involving Angpt-2 regulated proteins, like ICAM-1 [32], the regulation might involve the same underlying BIFO mechanism on Angpt-2 which was not further investigated in that context and therefore, remains speculative but possible.

This study has limitations. First of all, it is hypothesis generating in nature and the *in vitro* findings require further *in vivo* confirmation. Secondly, the underlying mechanism of BIFO's effect on Angpt-2 in the endothelium remains unknown and it is unclear if BIFO might hold promise as a potential therapeutic strategy against injurious Angpt-2 release in sepsis.

Conclusions:

A potential off-target effect of the approved anti-fungal BIFO on Angpt-2 release and synthesis was confirmed in a series of *in vitro* experiments after initial detection in an unbiased FDA drug library screening. Given the protective effect on vascular permeability, BIFO might hold promise as a modulator of the injurious host response to an infection.

Further *in vivo* studies are needed in order to confirm these findings in a living organism and to further translate them into clinical use.

Material And Methods:

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed for Angiopoietin-2 (Angpt-2) from endothelial cell culture supernatants and endothelial cell lysates with the commercial human Angiopoietin-2 DuoSet kit (DY623, R&D Systems, Minneapolis, MN). All additionally needed reagents were purchased from R&D Systems except for Normal Mouse Serum (NMS) (Jackson ImmunoResearch Laboratories, Westgrove, PA) and Bovine Serum Albumine (BSA) (Sigma-Aldrich, St. Louis, MO).

Cell culture studies

Primary endothelial cell isolation was performed according to institutional and governmental guidelines. In brief, human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins. HUVECs were isolated with heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA), Phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA) and Collagenase (Biochrom, Berlin, Germany). Then, they were grown to confluency in endothelial growth medium (EBM-2) containing 2% FBS according to manufacturer's instructions (Lonza, Basel, Switzerland). For HUVEC donation, informed consent was obtained and the protocol was approved by the local ethical committee of Hannover medical school (No. 1303–2012).

HUVECs were used in passages 3–5 and split with Trypsin/Ethylenediaminetetraacetic acid solution (Biochrom, Berlin, Germany), FBS and PBS.

Unless otherwise specified, HUVECs were stimulated with 10 µM Bifonazole (Sigma-Aldrich, St. Louis, MO), 10 ng/ml recombinant human TNF-alpha (R&D Systems, Minneapolis, MN), 50 ng/ml Phorbol-12-myristate-13-acetate (PMA) (Merck Millipore, Darmstadt, Germany), 10 µM DMSO for molecular biology (Sigma-Aldrich, St. Louis, MO), 10 µM Mibefradil dihydrochloride hydrate (Sigma-Aldrich, St. Louis, MO), 50 µM TTA-A2 (Sigma-Aldrich, St. Louis, MO), 1 mM N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-Name) (Sigma-Aldrich, St. Louis, MO) and 1 U/ml Thrombin (Merck Millipore, Darmstadt, Germany). Each condition was reproduced for n = 4–10 times.

Antibodies and reagents

All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified. Antibodies against Angpt-2 (AF623) (R&D Systems, Minneapolis, MN), vWF (A008202) (Agilent Dako, Santa Clara, CA), VE-Cadherin (555661) (BD Pharmingen, San Jose, CA), Phalloidin/F-Actin (A22283) (Invitrogen, Carlsbad, CA) were utilized. As secondary antibodies goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, CA) were used.

Fluorescent immunocytochemistry

Coverslips were coated with collagen (Sigma-Aldrich, St. Louis, MO) and HUVECs were grown to confluency. Then coverslips were fixed with a 1:1 solution of Aceton (Thermo Fisher Scientific, Waltham, MA) and Methanol (Th. Geyer Hamburg, Hamburg) or 2% Paraformaldehyde (Th. Geyer Hamburg, Hamburg). All coverslips were then blocked with 10% donkey serum (Jackson Immuno Research Inc., West Grove, PA) and those fixed with 2% Paraformaldehyde were additionally permeabilized with 0.1% Triton X-100 in PBS (Sigma-Aldrich, St. Louis, MO). The primary antibody was incubated for 1 h at room temperature, followed by washing with Phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA). The secondary antibody was incubated for 1 h at room temperature. Alexa Fluor 488 donkey anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA), Alexa Fluor 555 donkey anti-goat IgG (Thermo Fisher Scientific, Waltham, MA) and Alexa Fluor 556 donkey anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) were used as secondary antibodies.

Pictures were taken with a Leica DMI 6000B microscope and obtained with the same light exposure conditions and gain.

RNA –Isolation and quantitative PCR

RNA was isolated from cultured HUVECs using the RNeasy MicroKit (Qiagen, Hilden, Germany) following manufacturer's instructions. With the transcriptor First Strand cDNA Synthesis (Roche Diagnostics, Rotkreuz, Switzerland), 0.5 µg of total extracted RNA was reverse transcribed to cDNA. After that, SYBR Green real-time-PCR using a LightCycler 480II (Roche, Basel, Switzerland) was performed. The following primers were used: human ß-Actin (fv: CTG GAA CGG TGA AGG TGA CA, rev: AGT CCT CGG CCA CAT TGT G), human Ang-2 (fv: GCC GCT CGA ATA CGA TGA CT, rev: GCT TCA TTA GCC ACT GAG TGT TGT). For each sample triplicates of RT-qPCR were performed and average of the cycle values was formed.

Transendothelial electrical resistance (TER)

Special cell culture plates (ibidi, 8W10E) were coated with collagen for 1 h at 37 °C and HUVECs were grown at 37 °C. Monolayer confluency was determined regarding manufacturer's recommendation by electrical criteria (resistance > 1800 ohms and capacitance > 10 nF). HUVECs were stimulated with 10 µM BIFO for 24 h. Then 1 U/ml Thrombin was applied. Medium was changed by carefully removing the medium and renewing it by not disturbing the cell monolayer in these steps. Transendothelial electrical resistance (TER) was measured at different time points by an electric cell-substrate impedance sensing system (ECIS, Applied BioPhysics Inc.). Values were either plotted over time or as bar graphs at time points of maximal response as described elsewhere [37, 38]. Resistances of each condition at each time point were divided by its condition starting resistance to calculate normalized TER.

Transwell Assay

HUVECs were grown to confluency in 24-well plates corning 6.5 mm Transwell inserts with 0.4 µm polycarbonate membranes in the upper chambers (inserts) (18312002) (Corning Incorporated, Corning, NY). Inserts were prepared with medium and flow-through was collected in order to check membranes. If no flow-through was found, inserts were put onto a new 24-well plate and HUVECs were treated with BIFO for 1 h. Then HUVECs were treated with 1 U/ml Thrombin for 24 h. Streptavidin-horseradish peroxidase (HRP) was added to the upper chamber and flow-through was collected from the lower chamber at indicated time points. Leakage of cell monolayers was quantified by the concentration of HRP in the lower chamber by photometric reading at 450 nm. Further details on measuring leakage with a Transwell-Assay are described elsewhere [39].

Statistical Analysis

We used GraphPad Prism5 (La Colla, CA) for data analysis and graph generation. Data was tested for Gaussian distribution with Kolmogorov-Smirnov-test. When data showed Gaussian distribution unpaired t-test with Welsh's correction was used for comparison of two independent groups or One-Way-ANOVA with Bonferroni post test was used for comparison of more than two groups. When data did not show Gaussian distribution Mann-Whitney U test was used for comparison of two independent groups or One-Way-ANOVA with Dunn's post test was used for statistical comparison of more than two independent groups. Results were seen as significant for $p < 0.05$. Columns are presented as mean ± SD.

List Of Abbreviations:

Angpt-1 Angiopoietin-1

Angpt-2 Angiopoietin-2

BIFO Bifonazole

DAPI 4',6-diamidino-2-phenylindole

FDA U.S. Food and Drug Administration

HRP Streptavidin-horseradish peroxidase

HUVEC Human umbilical vein endothelial cells

ICAM-1 intercellular adhesion molecule-1

L-Name N_{ω}-Nitro-L-arginine methyl ester hydrochloride

PMA Phorbol-12-myristate-13-acetate

TNFα Tumor necrosis factor α

Veh vehicle

vWF von-Willebrand factor

Methods

ECIS Electric cell-substrate impedance sensing system

ELISA Enzyme-linked immunosorbent assay

qPCR quantitative polymerase chain reaction

TER Transendothelial electrical resistance

Declarations:

Ethics Approval and consent to participate:

From all HUVEC donors, informed consent was obtained and approved by the ethical committee of Hannover medical school (No. 1303 - 2012).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TP designed and performed experiments, analyzed and discussed data and co-wrote the manuscript. TOI designed and performed experiments and discussed data. VME performed experiments and discussed data. KS and HH discussed data. SD designed the project, analyzed and discussed data and co-wrote the manuscript. All authors read and approved the final manuscript.

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Footnotes

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Figures

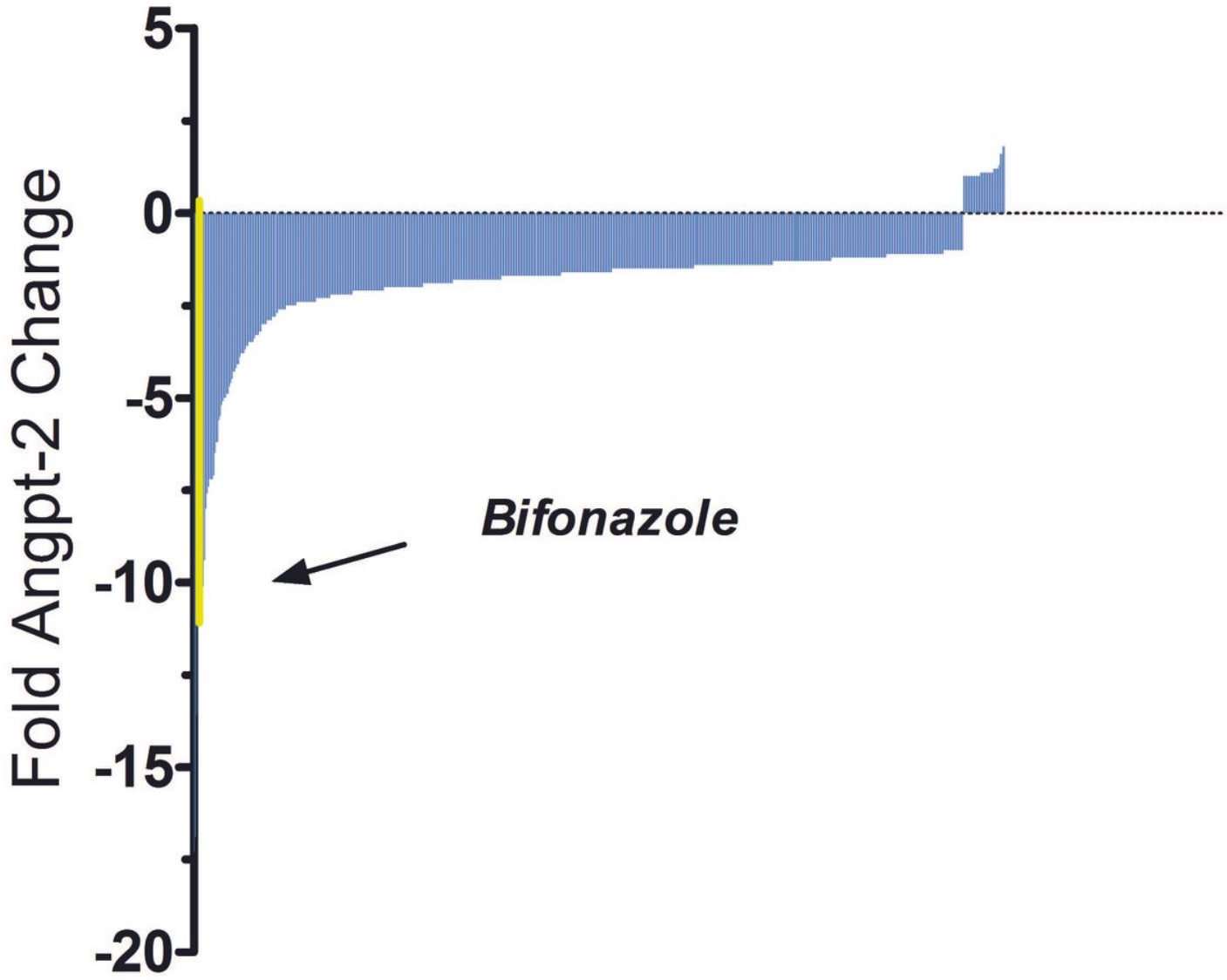

Figure 1

Identification of Bifonazole as a possible suppressor of Angiopoietin-2 Human umbilical vein endothelial cells (HUVECs) were grown in 96-well-plates and stimulated for 24 hours with different Food and Drug Administration (FDA)-approved drugs. Angiopoietin-2 (Angpt-2) release in the supernatants was quantified by Enzyme-linked immunosorbent assay (ELISA). The results are shown as a fold change of the mean value and sorted from strongest inhibitor (left) to strongest inductor (right).

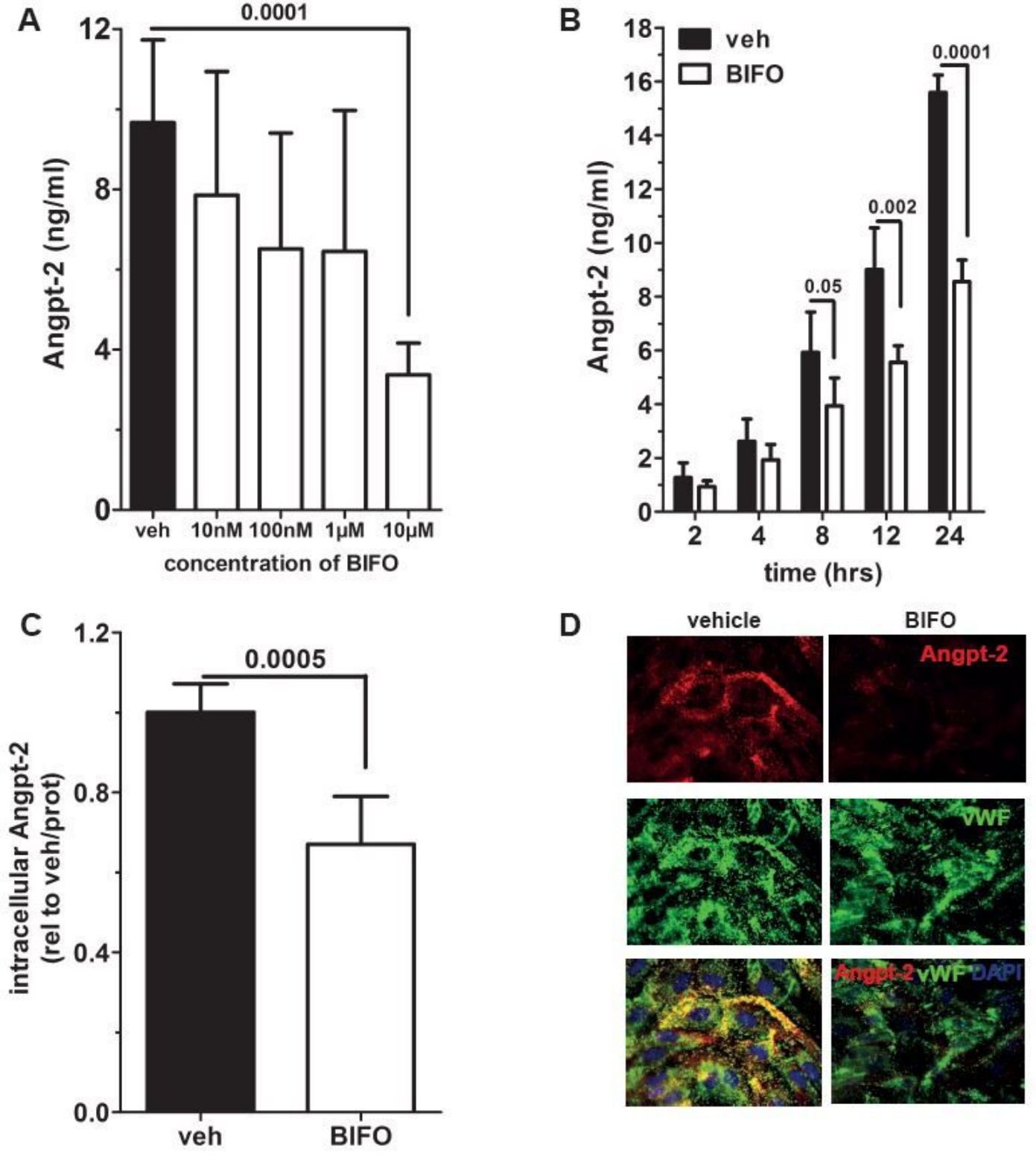

Figure 2

Bifonazole reduces baseline Angiopoietin-2 (A) Human umbilical vein endothelial cells (HUVECs) were treated with different concentrations of Bifonazole (BIFO) for 24 hours. Angiopoietin-2 (Angpt-2) was measured in the supernatant by Enzyme-linked immunosorbent assay (ELISA) (n = 4 - 8). (B) HUVECs were treated with 10 μM BIFO or vehicle (veh) for the indicated amount of time. Angpt-2 in the supernatant was quantified by ELISA (n = 5 - 6). (C) Angpt-2 was measured in cell lysates by ELISA after

24 hours treatment with 10 µM BIFO or veh (n = 5 - 6). (D) HUVECs were grown to confluency and stimulated with either 10 µM BIFO or veh for 24 hours. Fluorescent immunocytochemistry for Angpt-2 (red), von-Willebrand factor (vWF, green) and 4',6-diamidino-2-phenylindole (DAPI, blue) was performed (n = 5). Representative pictures were taken with a 63x objective. Columns are presented as mean ± SD.

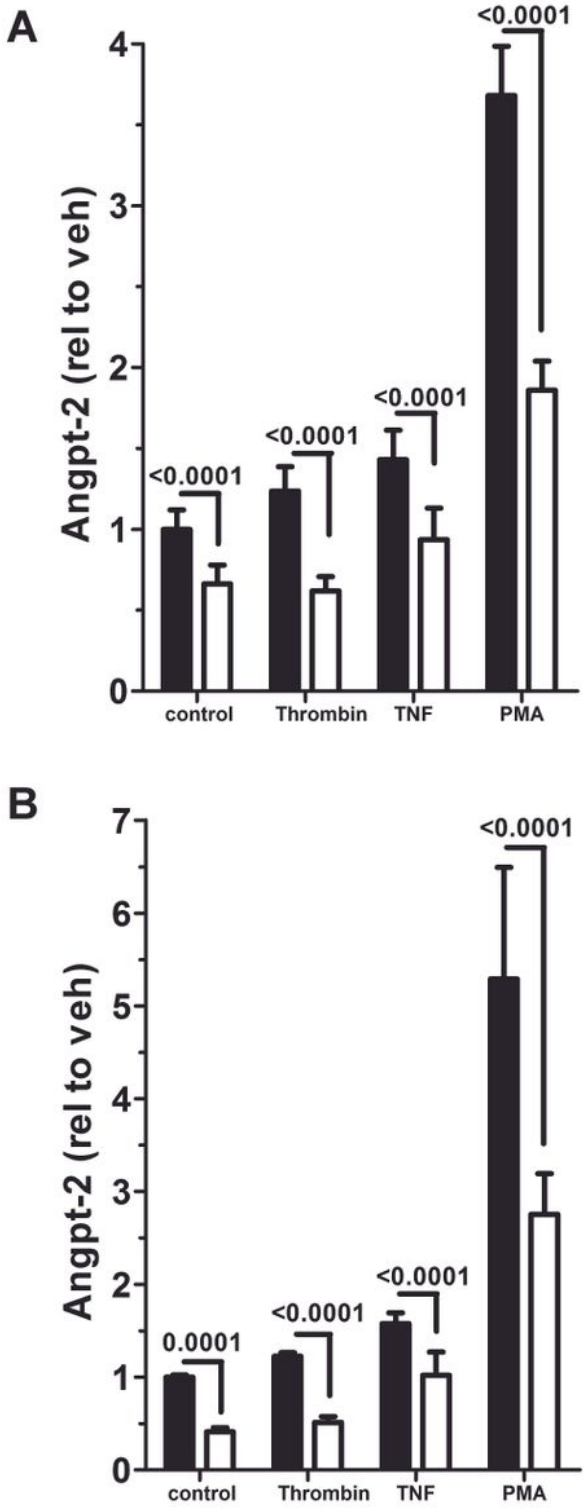

Figure 3

Bifonazole reduces Angiopoietin-2 after stimulation 10 ng/ml tumor necrosis factor α (TNFα), 1 U/ml Thrombin, 50 ng/ml Phorbol-12-myristate-13-acetate (PMA) or control were applied to human umbilical vein endothelial cells (HUVECs) for (A) 12 hours or (B) 24 hours after pretreatment with 10µM Bifonazole (BIFO) or vehicle (veh) for 1 hour (n = 5 - 12). Angiopoietin-2 (Angpt-2) was measured in the supernatants by ELISA. Columns are presented as mean ± SD.

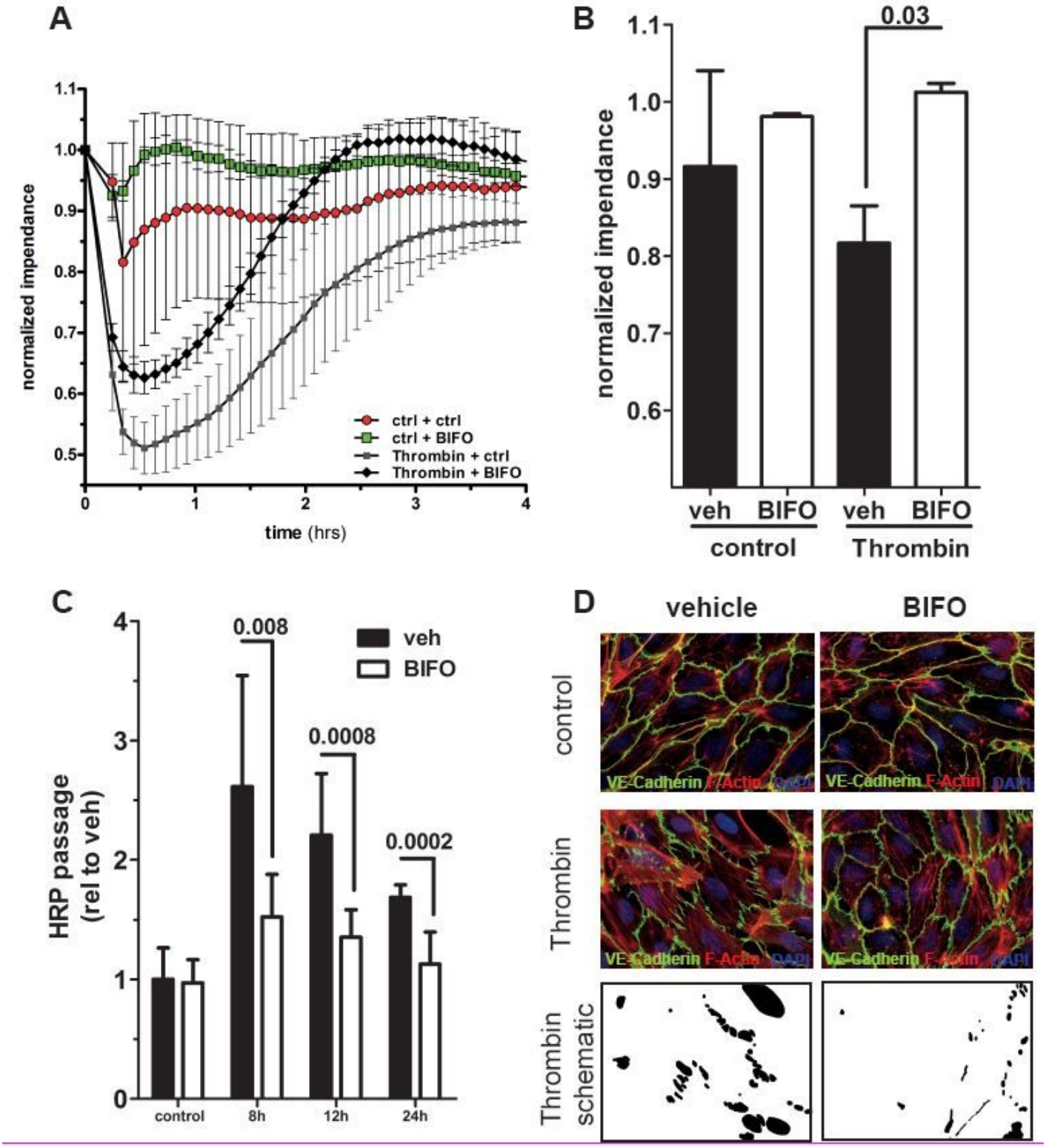

Figure 4

Functional barrier improvement through Bifonazole (A) Human umbilical vein endothelial cells (HUVECs) were pretreated with 10μM Bifonazole (BIFO) or vehicle (veh) for 24 hours. 1 U/ml Thrombin or control was applied (n = 2 - 4). Transendothelial electrical resistance (TER) was measured at different time points by an electric cell-substrate impedance sensing (ECIS) system. (B) Normalized impedance of HUVECs treated as described above is shown for the time point of maximum response to stimulation with 1 U/ml Thrombin or control (n = 2 - 4). (C) HUVECs were pretreated with 10 μM BIFO or veh for 1 hour and stimulated with 1 U/ml Thrombin for 24 hours. Flow-through was collected after 8, 12 and 24 hours and quantified by reading of optic density for Streptavidin-horseradish peroxidase (HRP) (n = 9). (D) HUVECs were grown to confluency and pretreated with 10 μM BIFO or veh for 24 hours. 1 U/ml Thrombin or control were applied for 1 hour. Fluorescent immunocytochemistry for VE-Cadherin (green), F-Actin (red) and 4',6-diamidino-2-phenylindole (DAPI, blue) was performed (n = 4). Leakage points between adjacent cells are schematically shown in the two lower panels. Representative pictures were taken with a 63x objective. Columns are presented as mean ± SD.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplemental2.pdf
- Supplemental1.pdf