Angiotensin II increases the permeability and PV-1 expression of endothelial cells

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Submitted 29 April 2011; accepted in final form 17 October 2011

Am J Physiol Cell Physiol 302: C267–C276, 2012. First published October 19, 2011; doi:10.1152/ajpcell.00138.2011.—Angiotensin II (ANG II), the major effector molecule of the renin-angiotensin system (RAS), is a powerful vasoactive mediator associated with hypertension and renal failure. In this study the permeability changes and its morphological attributes in endothelial cells of human umbilical vein (HUVECs) were studied considering the potential regulatory role of ANG II. The effects of ANG II were compared with those of vascular endothelial growth factor (VEGF). Permeability was determined by 40 kDa FITC-Dextran and electrical impedance measurements. Plasmalemmal vesicle-1 (PV-1) mRNA levels were measured by PCR. Endothelial cell surface was studied by atomic force microscopy (AFM), and caveolae were visualized by transmission electron microscopy (TEM) in HUVEC monolayers. ANG II (10−7 M), similarly to VEGF (100 ng/ml), increased the endothelial permeability parallel with an increase in the number of cell surface openings and caveolae. AT1 and VEGF-R2 receptor blockers (candesartan and ZM-323881, respectively) blunted these effects. ANG II and VEGF increased the expression of PV-1, which could be blocked by candesartan or ZM-323881 pretreatments and by the p38 mitogen-activated protein (MAP) kinase inhibitor SB-203580. Additionally, SB-203580 blocked the increase in endothelial permeability and the number of surface openings and caveolae. In conclusion, we have demonstrated that ANG II plays a role in regulation of permeability and formation of cell surface openings through AT1 receptor and PV-1 protein synthesis in a p38 MAP kinase-dependent manner in endothelial cells. The surface openings that increase in parallel with permeability may represent transcellular channels, caveolae, or both. These morphological and permeability changes may be involved in (patho-) physiological effects of ANG II.

VASCULAR ENDOTHELIUM is involved in diverse physiological functions such as blood/interstitium fluid exchange control. Fenestrae and caveolae formation are among several mechanisms by which endothelium regulates permeability. Fenestrae, cell surface windows that cut through the endothelial cell body, with openings of 60–70 nm in diameter (in liver sinusoids and glomeruli about 250 nm) facilitate the exchange between blood and interstitium (7). Caveolae are small, flask-shaped cell surface invaginations with diameters of 55– 65 nm. Caveolin, a 21-kDa protein, is the crucial component of caveolae (27, 33). Functionally, caveolae have been implicated in endocytosis, potocytosis, and transcytosis (26). Frequently, caveolar openings and fenestrae contain diaphragms, except for glomerular capillaries, afferent arteriole, and the sinusoids of the liver (31, 43, 45). Diaphragms are composed of radial fibrils anchored at the rim and interwoven in a central knob. The component of these fibrils is the plasmalemmal vesicle-1 (PV-1) protein, a 60-kDa, single-span, cationic, type II transmembrane glycoprotein that forms homodimers (38, 39). It was suggested that diaphragms located in the openings of caveolae and fenestrae function as a sieve, permitting a fine tuning of permeability for these structures (11). Recently, further important functions have been attributed to PV-1 like facilitating the transcellular migration of the immune cells through the endothelial cell layer (19). In vivo studies showed that PV-1 expression is correlated with disruption of blood-brain barrier in the ischemic brain and with microvascular leakage in diabetic retinopathy (36, 37).

Angiotensin II (ANG II), a vasoactive molecule, is involved in hypertension and renal failure, among others. ANG II can stimulate the release of other vasoactive and proinflammatory cytokines (35). ANG II increased leukocyte rolling, adhesion, and migration in rat mesenteric postcapillary venules (1, 29). In vivo studies showed that PV-1 expression is correlated with disruption of blood-brain barrier in the ischemic brain and with microvascular leakage in diabetic retinopathy (36, 37).

Vascular endothelial growth factor (VEGF) belongs to a family of dimeric glycoproteins that includes VEGF-A to -E and placenta growth factor (PLGF). VEGF-A has five different isoforms, VEGF165 being the most abundant and biologically active form of it, here referred to as VEGF (23). Constitutive VEGF expression was shown in epithelial cells adjacent to fenestrated endothelium of choroid plexus and kidney glomeruli (2, 3). VEGF acts by binding to receptor tyrosine kinases (VEGFR-1 and -2) leading to the activation of various signal transduction molecules, including p38 MAPK (20, 28), which itself belongs to the stress-activated serine/threonine protein kinases. This kinase is activated by phosphorylation through the upstream kinases MKK3 and MKK6 (25). Activation of p38 is involved in endothelial cell responses as migration, permeability, and cell survival (21, 34) and regulates the stability of different mRNAs by activation of MAPKAP2 (MK2) (22). VEGF, as a potent permeability enhancing agent,
induces fenestrae formation in vivo and in vitro (10, 30) and also increases PV-1 expression (40).

We have previously demonstrated in vivo that renal arterial renin activity may alter permeability and fenestration of the juxtaglomerular afferent arteriolar endothelium (32). In this study we hypothesized that ANG II may play a role in the induction of caveolae and expression of PV-1, hence leading to permeability change in endothelium of human umbilical vein. Primary cell cultures of umbilical endothelium were utilized to study the effect of ANG II compared with VEGF. Transmission electron microscopy was used to characterize the morphology of the caveolae and atomic force microscopy to follow the changes in their density. Permeability of the endothelial monolayer was determined by measuring the exchange of labeled dextran and recording the electrical impedance. The variations in PV-1 expression were followed by real-time PCR and immunocytochemistry. Our data shows that ANG II regulates permeability, PV-1 expression, and caveolae formation in a dose- and time-dependent manner. These effects are mediated through AT1 receptor and p38 MAPK and are very similar to those of VEGF.

MATERIALS AND METHODS

Endothelial Cell Isolation and Culture

Endothelial cells were isolated from human umbilical cord veins according to Jaffe et al. (17). Briefly, human umbilical vein endothelial cells (HUVEC) were separated by collagenase treatment (Sigma, St. Louis, MO). Cells were seeded onto 0.5% gelatin-coated flasks (Sigma) and cultured in M199 medium (Sigma) supplemented with 15% fetal bovine serum (FBS, GIBCO/Life Technologies, Burlington, ON, Canada), 100 IU/ml penicillin (GIBCO), 100 µg/ml streptomycin (GIBCO), 7.5 IU/ml heparin (Merckle, Ulm, Germany), 2 ng/ml epidermal growth factor (R&D Systems, Abington, UK), and 250 pg/ml β-endothelial cell growth factor (R&D Systems), referred to as complete medium. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Confluent cells were harvested with 0.05% trypsin-EDTA (GIBCO) for subcultures. Cells from passages 2–4 were used for experiments.

Assessment of Endothelial Permeability In Vitro

Cells were grown on gelatin-fibronectin-coated MilliCell culture plate inserts (polycarbonate membranes with pore size: 0.4 µm, Millipore, Carrigtwohill, Ireland) in phenol red-free M199 complete medium for 4–5 days to achieve confluence. For measurements, complete medium was replaced with a medium containing 5% FBS, and cells were treated with 10⁻⁷ M ANG II or 100 ng/ml VEGF or vehicle. After 48 h of treatment endothelial cells were fixed with Zamboni fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for 1 h at room temperature. Fixative was washed out carefully and cells were treated with 1% OsO₄ for 1 h, dehydrated in ethanol, stained with 1% uranyl acetate in 50% ethanol for 1 h, and embedded in Taab 812 (Taab Laboratories Equipment). Ultrathin sections were cut against the membrane, using a Leica UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany). Digital images were obtained using a Hitachi 7100 electron microscope (Hitachi, Japan), brightness and contrast being adjusted if necessary using Adobe Photoshop CS3 (San Jose, CA). For morphometric analysis randomly acquired images were used at magnifications of ×30,000 with 7.5 × 7.5 µm picture area. All experiments were repeated three times.

Visualization of Endothelial Cell Surface by Transmission Electron Microscopy

For electron microscopy cells were grown on gelatin-fibronectin-coated MilliCell culture plate inserts in M199 complete medium for 4–5 days to achieve confluence. For measurements, complete medium was replaced with a mixture containing 5% FBS, and cells were treated with 10⁻⁷ M ANG II or 100 ng/ml VEGF or vehicle. After 48 h of treatment endothelial cells were fixed with Zamboni fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for 1 h at room temperature. Fixative was washed out carefully and cells were treated with 1% OsO₄ for 1 h, dehydrated in ethanol, stained with 1% uranyl acetate in 50% ethanol for 1 h, and embedded in Taab 812 (Taab Laboratories Equipment). Ultrathin sections were cut against the membrane, using a Leica UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany). Digital images were obtained using a Hitachi 7100 electron microscope (Hitachi, Japan), brightness and contrast being adjusted if necessary using Adobe Photoshop CS3 (San Jose, CA). For morphometric analysis randomly acquired images were used at magnifications of ×30,000 with 7.5 × 7.5 µm picture area. All experiments were repeated three times.

Measurement of Electrical Impedance as an Index of Endothelial Cell Permeability

The permeability changes of endothelial cells to ANG II or VEGF treatments were measured in real time using an electric cell-substrate impedance sensing (ECIS) system (Applied BioPhysics, Troy, NY) as described previously (41). Cells were grown to confluence on gelatin-fibronectin-coated ECIS culture wares (SwIE). Each well contained a gold microelectrode with a diameter of 250 µm and a reference electrode. Electrodes were placed in an incubator (37°C, 5% CO₂) and connected to an ECIS model 1600 apparatus. Media containing 5% FBS were used for experimental treatments and after the equilibration period of 2 h, cells were treated with 10⁻⁷ M ANG II or 100 ng/ml VEGF or vehicle. During measurements, electrical impedance was monitored in real time for 48 h after addition of ANG II or VEGF. Monolayers were checked by light microscopy for integrity before and after impedance measurements. Monolayer impedance was normalized at time 0.

Visualization of Endothelial Cell Surface by Atomic Force Microscopy

Endothelial cells were grown on gelatin-fibronectin-coated glass coverslips and allowed to reach confluence when complete medium was replaced with a medium containing 5% FBS. Cells were then treated with various doses of ANG II (Sigma) or VEGF (R&D Systems) or vehicle. For atomic force microscopy (AFM) studies cells were fixed by dehydration through increasing grades of ethanol. Apical surface membrane was scanned.

AFM-derived data were evaluated with Scanning Probe Image Processor software (SPIP, Image Metrology, Horsholm, Denmark). Single cell surface areas (8 × 8 µm) were scanned and each experimental group was repeated at least three to four times (coverslips). On each coverslip, at least three different cells were examined and two to four scans were made. The results show the average of all scanned areas.

The AFM was originally assembled by the University of Twente (Enschede, The Netherlands) (18). In this microscope, the piezoelectric scanner drives directly the cantilever instead of the sample. Therefore, conventional glass microscopic slides mounted on a Zeiss Axioshot (Zeiss, Oberkochen, Germany) inverted microscope was used for localization and selection of cells. Coverslips were fixed onto glass slides and imaged with silicon nitride tips (Park Scientific, Sunnyvale, CA) having nominally 20 nm radius of curvature, or Ultralever high-aspect ratio silicon tips (~10 nm of radius). Intermit-
tent-contact mode was used with “F”-type microlevers or “D”-type ultralevers at a few tenths of a kilohertz below their resonant frequency (around 120 and 160 kHz, respectively). In contact-mode imaging, we collected 512 × 512 pixel images in height and error signal modes. The scanned images were not corrected for tip convolution, but all samples from a given experiment were measured with the same tip. Figure 4 images were made at the Department of Biophysics, Semmelweis University. Noncontact mode AFM images were acquired with an Asylum Research MFP3D instrument (Santa Barbara, CA) using silicon-nitride cantilevers (Olympus AC160 cantilever, resonance frequency in air ~330 kHz). Images (512 × 512-pixels) were collected at a typical scanning frequency of 0.3–0.7 Hz and with a set point of 0.3–0.8 V.

Assessment of PV-1 mRNA Expression by Real-Time PCR

Endothelial cells were grown on gelatin-coated six-well plates and allowed to reach confluence when complete medium was replaced with a medium containing 5% FBS. Cells were then treated with various doses of ANG II or 100 ng/ml VEGF for 24 and 48 h. RNAs were isolated using GenElute Mammalian Total RNA Kit (Sigma) and later transcribed to cDNAs with TaqMan Reverse Transcription kit (Applied Biosystems) on a Bio-Rad iCycler thermal cycler. PV-1 real-time PCR was performed using a TaqMan assay published previously (40) in a Bio-Rad CFX96 thermal cycler. Data were normalized to ribosomal RNA content (TaqMan Ribosomal RNA control reagents, Applied Biosystems, Forster City, CA), relative quantitation being performed using the Bio-Rad CFX Manager Software v. 1.7. In each experiment three parallel points were examined, and the numbers of individual experiments are shown in the figure legends.

Western Blot

After treatments, cells cultured on six-well plates were scraped in Triton lysis buffer containing 1% Triton X-100, 50 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EGTA, and 50 mM NaF in distilled water. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL). Cell lysates were mixed 1:1 in 2× Laemml sample buffer containing 2-mercaptoethanol and boiled for 5 min. Equal amounts of protein were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked in Tris-buffered saline and 0.1% Tween 20 containing 5% nonfat dry milk. Antibody against phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, Danvers, MA) was used. Data were normalized to α-tubulin with the appropriate primary antibody (Sigma). Bands were visualized with Amersham ECL Western blotting detection kit (GE Healthcare, Little Chalfont, UK), and Kodak Biomax XAR (Kodak) photo films were exposed for different time lengths. Experiments were repeated three times and specific Western blot images are shown.
**Immunocytochemistry**

After 48 h of $10^{-7}$ M ANG II or 100 ng/ml VEGF or vehicle treatments, cells grown onto glass coverslips were washed with PBS containing Ca$^{2+}$ and Mg$^{2+}$ and fixed with 4% paraformaldehyde (PFA) for 30 min. PFA-fixed cells were permeabilized in PBS containing 0.1% Triton X-100 and blocked in 5% BSA solution for 1 h. Then cells were incubated with different primary antibodies, namely, PV-1 (PAL-E, Santa Cruz, Santa Cruz, CA), caveolin-1 (Upstate, Temecula, CA), and VE-cadherin (Cell Signaling, Beverly, MA) overnight, followed by 1 h incubation with the adequate fluorescent secondary antibodies. PV-1 was visualized with Alexa488 conjugated anti-mouse antibody and caveolin-1 with Alexa594 conjugated anti-rabbit secondary antibody (both from Molecular Probes/Invitrogen, Eugene, OR). For detection of VE-cadherin we used NL-493 conjugated anti-rabbit antibody (Northern Lights, R&D Systems, Minneapolis, MN). The coverslips were mounted using antifade reagent ProLong Gold with DAPI (Molecular Probes/Invitrogen). Fluorescent images were captured using a DMR fluorescence microscope (Leica Microsystems).

**Treatments**

**Angiotensin II treatments.** For AFM studies, the effect of ANG II on HUVEC (in M199 medium containing 5% FBS) was studied. Cells were treated for 48 h with ANG II in a final concentration of $10^{-8}$ M (10 μg/ml), $10^{-6}$ M (1 μg/ml), $10^{-7}$ M (100 ng/ml), and $10^{-8}$ M (10 ng/ml). At the end of the treatments, cells were prepared for AFM studies.

Where indicated, the AT1 receptor blocker candesartan (AstraZeneca, Mölndal, Sweden) ($10^{-5}$ M), and the p38 MAP kinase inhibitor SB-203580 (Calbiochem, EMD Biosciences, San Diego, CA) (10 μM) were added onto the cells in separate experiments 30 min before ANG II treatments.

For permeability measurements, HUVEC’s grown on MilliCell chambers were treated for 48 h with the same concentration of ANG II and candesartan as in the AFM experiments.

**VEGF treatments.** For AFM studies, the effect of VEGF on HUVEC (in M199 medium containing 5% FBS) was studied. Cells were treated for 48 h with VEGF in final concentration of 10 ng/ml (2.6 × $10^{-10}$ M), 25 ng/ml (6.5 × $10^{-10}$ M), and 100 ng/ml (2.6 × $10^{-9}$ M). At the end of the treatments, cells were prepared for AFM studies.

Where indicated, the VEGFR-2 receptor blocker ZM-323881 (50 nM) (synthesized at Budapest University of Technology and Economics, Faculty of Chemical and Bioengineering, Hungary) and the p38 MAP kinase inhibitor SB-203580 (10 μM) were added on to the cells 30 min before VEGF treatment.

For permeability measurements, HUVEC’s grown on MilliCell chambers were treated for 48 h with the same concentration of VEGF and ZM-323881 as in the AFM experiments.

**Ethical Issues**

All experimental protocols have been approved by the Ethical Committee of the Semmelweis University, Budapest.

**Statistical Analysis**

All data are presented as means ± SE. Data were analyzed by one-way ANOVA, followed by LSD test. Significance was accepted at $P < 0.05$.

**RESULTS**

**ANG II and VEGF Increased the Permeability of HUVEC Monolayers**

To investigate the effects of ANG II on HUVEC monolayer permeability, cells were treated with $10^{-5}$ M, $10^{-6}$ M, $10^{-7}$ M, or $10^{-8}$ M doses of ANG II for 48 h. ANG II at $10^{-7}$ M significantly increased endothelial permeability measured by 40 kDa FITC-Dextran (control: $13.2 \times 10^{-6}$ cm/s ± 0.5 × $10^{-6}$; ANG II: $15.1 \times 10^{-6}$ cm/s ± 0.7 × $10^{-6}$, $P < 0.05$) (Fig. 1A, left).

The role of AT1 receptors in ANG II-induced increased permeability was studied. HUVEC monolayers were pretreated with $10^{-5}$ M candesartan for 30 min before 48 h treatment with $10^{-7}$ M ANG II. Candesartan significantly blunted the increased permeability response (ANG II: $15.9 \times 10^{-6}$ cm/s ± 1.3 × $10^{-6}$, candesartan + ANG II: $10.2 \times 10^{-6}$ cm/s ± 1.1 × $10^{-6}$, $P < 0.05$) (Fig. 1A, right).

For comparison, HUVEC monolayers were treated with 10, 25, or 100 ng/ml doses of VEGF for 48 h. VEGF at 100 ng/ml could significantly increase the endothelial permeability (control: $12.2 \times 10^{-6}$ cm/s ± 0.7 × $10^{-6}$; 100 ng/ml VEGF: $16.1 \times 10^{-6}$ cm/s ± 0.5 × $10^{-6}$, $P < 0.01$) (Fig. 1B, left).

To investigate the role of VEGFR-2 receptors in VEGF-induced permeability change, HUVEC monolayers were pretreated with 50 nM ZM-323881 for 30 min, before 48 h treatment with 100 ng/ml VEGF. ZM-323881 significantly inhibited permeability response induced by VEGF (VEGF: $16.4 \times 10^{-6}$ cm/s ± 0.7 × $10^{-6}$; ZM-323881 + VEGF: $12.9 \times 10^{-6}$ cm/s ± 1.2 × $10^{-6}$, $P < 0.05$) (Fig. 1B, right).

The electrical impedance of HUVEC monolayers with ECIS system was measured and used as an indicator of permeability change. Impedance measurements went on for 2 h to reach a steady state before the treatments with ANG II ($10^{-7}$ M) or VEGF (100 ng/ml) started, which caused a continuous decrease in electrical impedance unlike changes in the control. The electrical impedance in ANG II- and VEGF-treated groups compared with baseline at 48 h was significantly decreased (ANG II: 0.83 ± 0.03; $P < 0.05$; VEGF: 0.71 ± 0.04; $P < 0.01$). (Fig. 2.)

**Visualization and Morphology of HUVEC With TEM After ANG II Treatment**

Possible morphological changes in the HUVEC monolayer as the result of ANG II treatment were studied. Transmission
electron microscopy (TEM) of ANG II- (10^{-7} M) or VEGF (100 ng/ml)-treated (48 h) monolayers showed increased number of caveolae. In control cells (Fig. 3) few caveolae are found at the basal cell membrane and in the surrounding cytoplasm. The 48-h long ANG II (Fig. 3B) and VEGF (Fig. 3C) treatments induced an increase in the accumulation of caveolae both at the cell membrane and in the cytoplasm. Some of these vesicles fused and formed tubulovesicular-like structures. Morphometry displayed significant increase in the number of vesicular/caveolar structures (4.4 ± 0.6 for control, 9.4 ± 1.1 for ANG II-treated group and 13.2 ± 2.0 for VEGF-treated group, P < 0.01, n = 30 pictures) calculated on 7.5-μm wide sections. No change in the diameter of these vesicular/caveolar structures was registered (57.9 μm ± 1.8 for control, 58.4 μm ± 1.4 for ANG II-treated group, and 60.3 μm ± 1.9 for VEGF-treated group).

**ANG II Treatment Increased the Number of Openings on the Surface of HUVECs**

AFM was employed to further characterize the cell surface changes. Cells were incubated with ANG II or VEGF for 48 h. Images of cell surfaces from 10^{-7} M ANG II-treated or 100 ng/ml VEGF-treated samples showed a porous surface compared with the control surfaces (Fig. 4). Evaluation of measured data with SPIP software revealed that 10^{-7} M ANG II significantly increased the number of invaginations in the 8 × 8 μm scanned area of cell surface (13.2 ± 1.1 in vehicle, 31.3 ± 4.0 in ANG II treated) (Fig. 5A, left). This significant increase was mainly due to the increase in the number of invaginations with diameters of up to 100 nm (10.4 ± 1.1 for control vs. 26.9 ± 3.8 for ANG II-treated cells).

Thirty minutes before 10^{-7} M ANG II treatments, HUVEC monolayers were pretreated with 100 ng/ml candesartan. After 48 h of cotreatment, candesartan significantly decreased the number of openings induced by ANG II (ANG II: 30.8 ± 3.3 vs. candesartan + ANG II: 11.3 ± 1.3, P < 0.01) (Fig. 5A, right).

For comparison, cells were treated with VEGF (10 ng/ml, 25 and 100 ng/ml) for 48 h. VEGF at 100 ng/ml induced a significant increase in the number of openings in 8 × 8 μm cell surface fields (11.5 ± 1.8 vs. 41.9 ± 5.8) (Fig. 5B, left). This significant change was mainly due to increase in the number of invaginations with diameters up to 100 nm (8.6 ± 1.6 vs. 34.8 ± 5.3).

To study the role of VEGFR-2 receptors, HUVEC monolayers were pretreated with 50 nM ZM-323881, 30 min before 100 ng/ml VEGF treatments. After 48 h of cotreatment, ZM-323881 significantly inhibited the effects of VEGF on the formation of cell surface invaginations (VEGF: 39.5 ± 5.2, ZM323881 + VEGF: 9.3 ± 2.4, P < 0.01) (Fig. 5B, right).

**ANG II and VEGF Phosphorylate p38 MAP Kinase in HUVECs**

The activation of p38 after 10^{-7} M ANG II or 100 ng/ml VEGF treatments was investigated by Western blot using antibody against the phosphorylated form of p38. Both treatments increased the phosphorylation of p38. VEGF induced a rapid and elevated activation of p38 with a peak after 5 min of treatment, and this effect was diminished after 60 min. The effect of ANG II was less compared with VEGF, and the activation was maximal after 30 min of treatment (Fig. 6).

**ANG II Increased the Expression of Diaphragm Protein PV-1 After 48 h**

The effects of ANG II and VEGF on PV-1 mRNA levels were determined. Forty-eight hours of ANG II (10^{-7} M) or VEGF (100 ng/ml) treatments induced a significant increase in PV-1 mRNA (Fig. 7A). Some signaling steps involved in ANG II-dependent PV-1 mRNA expression were examined using...
specific inhibitors. Candesartan prevented ANG II (10^{-7} M)-dependent PV-1 mRNA expression. Inhibition of p38 MAP kinase by SB-203580 significantly blunted the effects of ANG II (Fig. 7B). Immunostaining for PV-1 was weak, almost negative in the controls, and revealed strong staining after ANG II and VEGF treatments. PV-1 staining following VEGF treatment shows some colocalization with caveolin-1, which is much less in ANG II-treated cells (Fig. 8).

**Inhibition of p38 MAP Kinase Prevents the Effects of ANG II and VEGF on the Number of Caveolae and Permeability in HUVEC**

Pretreatment (30 min) of HUVEC monolayer with SB-203580 (10 μM), a specific p38 MAP kinase inhibitor, prevented the ANG II- (10^{-7} M) and VEGF (100 ng/ml)-induced increase in the number of caveolae and endothelial permeability (Fig. 9).

**Effects of ANG II and VEGF on the Integrity of HUVEC Monolayers**

HUVEC monolayers were stained for VE-cadherin after 48 h treatment with 10^{-7} M ANG II or 100 ng/ml VEGF. Cells were tightly juxtaposed and stained for VE-cadherin at cell borders. In VEGF-treated cells, VE-cadherin staining was somewhat weaker, and a few intercellular openings were noticed. Nevertheless, the integrity of the cells were not disturbed (data not shown).

![Fig. 5. Cell surface density of caveolae, on 8 x 8 μm field, measured by AFM. A: left, ANG II effect on endothelial caveolae density (n = 4). Right, effect of AT1 receptor blockade on ANG II-induced increase in caveolae density (n = 3). B: left, VEGF effect on endothelial caveolae density (n = 3). Right, effect of VEGFR-2 receptor blockade on VEGF-induced increase in caveolae density (n = 3). **P < 0.01, ###P < 0.01.](http://ajpcell.physiology.org/)

![Fig. 6. Western blot analysis of p38 phosphorylation after treatments with 10^{-7} M ANG II or 100 ng/ml VEGF. Specific Western blot images are shown (n = 3).](http://ajpcell.physiology.org/)
mediated via AT1 receptor since application of AT1 receptor blocker (candesartan) inhibited these effects. VEGF (100 ng/ml) significantly increased the permeability of HUVEC monolayer via VEGFR-2 receptors. Real-time continuous recording of permeability changes of HUVEC monolayer (ECIS) showed a similar pattern to those of dextran-labeled permeability experiments. Impedance measurements revealed that permeability-enhancing effect of ANG II and VEGF appeared toward the last third period of recording.

Studies by TEM documented an increase in the density of caveolea and appearance of fused vesiculovacuolar structures in response to ANG II or VEGF treatments in line with permeability increase (Fig. 3). Compared with VEGF, ANG II effect on caveolea formation was less pronounced as its effect on permeability was smaller. Number and morphology of cell surface openings of membranous vesicles/invaginations were examined by AFM. The cell surface openings were almost round in shape and showed gyrus-like upfoldings. ANG II (10⁻⁷ M) and VEGF (100 ng/ml) increased the number of invaginations, mostly those whose diameter were below 100 nm. It was shown, similar to permeability change, that morphological alterations are also mediated via AT1 and VEGFR2 receptors, as specific receptor blockers inhibited these changes. These invaginations could represent caveolea or transcellular-fused vesicles (Fig. 3). Chen et al. (6) have also shown the transcellular characteristics of the fused caveolar vesicles (TEM) in VEGF-treated HUVEC monolayers parallel to increase in endothelial permeability (6). We have shown transcellular endothelial channels in the afferent arterioles in vitro (TEM) and in vivo (multiphoton microscopy) (32). Future studies will help clarify the exact nature of these invaginations.

After 48 h treatment, PV-1 expression, in response to ANG II and VEGF, showed a significant increase (real-time PCR), inhibited by AT1 and VEGFR-2 receptor blockers, respectively. This observation was confirmed by fluorescent microscopy, which showed increased PV-1 immunostaining after VEGF and ANG II treatments. PV-1 and caveolin-1 showed some colocalization in the treated cells. In previous studies it has been demonstrated that caveolea and clustered vesicular organelles are involved in the increase of transendothelial permeability (9, 11). Thus we assume that the fused caveolar vesicles providing a transcellular channel system for the movement of fluid and particles could partly contribute to the increase in endothelial permeability. Movement of fluid and particles can also occur via paracellular pathway. Immunostaining HUVEC monolayers for VE-cadherin after ANG II treatments revealed no considerable cellular change. This suggests that increase in the endothelial permeability, in response to ANG II, may occur mainly transcellularly.

Various cytokines exert their effects on permeability via p38 MAPK. p38 plays an important role in VEGF, transforming growth factor-β1, or thrombin-induced permeability increase, but its involvement in mediating ANG II-dependent effects was not addressed previously (5, 13, 16, 21). Earlier studies attributed the ANG II effects on permeability to the activation of cAMP/PKA signaling pathway or the elevation of intracellular Ca²⁺-levels (44, 46). Guo et al. (14) showed that ANG II induced the phosphorylation of p38 in HUVECs, but its involvement in permeability was not studied. Here we demonstrated that inhibition of p38 with SB-203580 abolishes the effects of ANG II and VEGF on

DISCUSSION

Vascular endothelial permeability is regulated by several mechanisms. The effects of ANG II have been studied in different vascular segments both in vivo (24, 42) and in vitro (12, 44, 46). This study examined the effects of ANG II on the permeability of HUVEC and its relation to p38 MAPK pathway. Results show that ANG II increases the permeability of HUVEC after 48 h of treatment with somewhat different pattern to that of VEGF.

The effects of ANG II and VEGF on permeability were parallel to morphological changes, namely increase in the number of caveolar vesicles. Both factors increased the expression of PV-1, a fenestrae- and caveolae-associated protein, in a p38-dependent manner.

ANG II (10⁻⁷ M) increased the permeability of HUVEC monolayers (Fig. 1A). Higher doses did not increase the permeability. Similar pattern of response of ANG II has also been observed in stimulation of sodium transport in renal proximal tubules (15) and in microvascular permeability of mesentery venules (42). This permeability-enhancing effect of ANG II is

Fig. 7. Levels of plasma membrane vesicle-1 (PV-1) mRNA measured by real-time PCR and normalized to ribosomal RNA levels. A: effect of different doses of ANG II treatments on the levels of PV-1 mRNA and compared with those of 100 ng/ml VEGF (n = 4). B: effects of AT1 receptor blockade or p38 inhibition on ANG II-induced PV-1 mRNA levels (n = 5). *P < 0.05, **P < 0.01, ###P < 0.001.
increasing endothelial permeability. This indicates that the permeability increase induced by ANG II, similarly to VEGF, is modulated via intracellular p38 MAP kinase-dependent signaling pathways.

It is known that p38 MAP kinase, beyond regulation of permeability, plays a role in the modulation of gene expression and organization of actin cytoskeleton. Strickland et al. (40) reported that PV-1 expression induced by VEGF is p38 MAP kinase dependent (40), whereas there was no data about the effect of ANG II on PV-1 expression. Here, we demonstrated that ANG II induced PV-1 expression, similarly to VEGF, also in a p38-dependent manner. Inhibition of p38 could therefore

![Fig. 8. Effects of ANG II or VEGF treatments on PV-1 localization. Representative image of HUVEC stained for caveolin-1 and PV-1 (separately and merged) in control, ANG II- or VEGF-treated group is shown. PV-1: green, caveolin-1: red.](image)

![Fig. 9. Effect of p38 mitogen-activated protein kinase (MAP) kinase blockade with SB-203580 on VEGF- or ANG II-induced increase in caveolae density (left) and permeability (right) (n = 3). **P < 0.01, #P < 0.05, ##P < 0.01.](image)
blunt the VEGF and ANG II effects on increasing the cell surface openings. This suggests that ANG II, similarly to VEGF, stimulates the expression of PV-1 and the increase in the density of cell surface openings via p38 activation.

In conclusion, the effect of ANG II in regulation of permeability and formation of cell surface openings and caveolea through AT1 receptor and PV-1 protein synthesis in a p38 MAP kinase-dependent manner in endothelial cells was reported as a new observation. The cell surface openings that increase in parallel with the permeability may represent fused vesiculovacuolar structures (functioning like a transcellular channel) or caveolae or both. These morphological and permeability changes may be considered as an additional mechanism in (patho-) physiological effects of ANG II.

ACKNOWLEDGEMENTS
We appreciate the help of Szabolcs Várboró from the Second Department of Obstetrics and Gynecology, Faculty of Medicine, Semmelweis University for providing umbilical cords.

GRANTS
These studies were supported by Hungarian Research Grants: OTKA AT 048767, ETT 564/2003, the Hungarian Kidney Foundation, Agency for Research Fund Management and Research Exploitation (KPI) Genomnanotech-DEBRET 06/2004, Botlyi fellowship (232/06, A.J.).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


