Angiopoietin-1 Opposes VEGF-Induced Increase in Endothelial Permeability by Inhibiting TRPC1-Dependent Ca\(^{2+}\) Influx

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Abstract—Angiopoietin-1 (Ang1) exerts a vascular endothelial barrier protective effect by blocking the action of permeability-increasing mediators such as vascular endothelial growth factor (VEGF) through unclear mechanisms. Because VEGF may signal endothelial hyperpermeability through the phospholipase C (PLC)-IP\(_3\) pathway that activates extracellular Ca\(^{2+}\) entry via the plasmalemmal store-operated channel transient receptor potential canonical-1 (TRPC1), we addressed the possibility that Ang1 acts by inhibiting this Ca\(^{2+}\) entry mechanism in endothelial cells. Studies in endothelial cell monolayers demonstrated that Ang1 inhibited the VEGF-induced Ca\(^{2+}\) influx and increase in endothelial permeability in a concentration-dependent manner. Inhibitors of the PLC-IP\(_3\), Ca\(^{2+}\) signaling pathway prevented the VEGF-induced Ca\(^{2+}\) influx and hyperpermeability similar to the inhibitory effects seen with Ang1. Ang1 had no effect on PLC phosphorylation and IP\(_3\) production, thus its permeability-decreasing effect could not be ascribed to inhibition of PLC activation. However, Ang1 interfered with downstream IP\(_3\)-dependent plasmalemmal Ca\(^{2+}\) entry without affecting the release of intracellular Ca\(^{2+}\) stores. Anti-TRPC1 antibody inhibited the VEGF-induced Ca\(^{2+}\) entry and the increased endothelial permeability. TRPC1 overexpression in endothelial cells augmented the VEGF-induced Ca\(^{2+}\) entry, and application of Ang1 opposed this effect. In immunoprecipitation studies, Ang1 inhibited the association of IP\(_3\) receptor (IP\(_3\)R) and TRPC1, consistent with the coupling hypothesis of Ca\(^{2+}\) entry. These results demonstrate that Ang1 blocks the TRPC1-dependent Ca\(^{2+}\) influx induced by VEGF by interfering with the interaction of IP\(_3\)R with TRPC1, and thereby abrogates the increase in endothelial permeability. (Circ Res. 2005;96:1282-1290.)

Key Words: endothelial permeability ■ vascular endothelial growth factor ■ angiopoietin-1 ■ Ca\(^{2+}\) influx ■ transient receptor potential channel

Vascular endothelial growth factor (VEGF) was originally described as vascular permeability factor based on its property of increasing vessel wall permeability.\(^1\) VEGF binds to VEGF receptor-2 (VEGFR2; aka Flk1) to stimulate phospholipase C (PLC)\(_{\gamma}\)-dependent IP\(_3\) production, thereby increasing the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)).\(^2\) The VEGF-induced rise in [Ca\(^{2+}\)]\(_i\) is the result of release of intracellular Ca\(^{2+}\) stores followed by extracellular Ca\(^{2+}\) entry. The Ca\(^{2+}\) influx portion of the response occurring via activation of plasmalemma Ca\(^{2+}\) channels was shown to be critical in signaling the increase in endothelial permeability in response to a variety of permeability-increasing mediators including thrombin.\(^3,4\) VEGF was shown to induce the formation of interendothelial junctional gaps and increase endothelial permeability through a Ca\(^{2+}\)-dependent pathway.\(^5-7\)

Ang1 cooperates with VEGF in the later stages of embryonic angiogenesis to form the mature vascular endothelial barrier.\(^8\) However, in the adult microvasculature, the binding of Ang1 to the Tie-2 receptor stabilizes endothelial cell interactions with the extracellular matrix and junctional proteins and enhances endothelial barrier function.\(^9,10\) Transgenic mice overexpressing Ang1 in dermal microvessels were resistant to the leakage of the albumin-binding Evans blue dye in response to VEGF and other inflammatory agents.\(^10\) Adenoviral-mediated delivery of Ang1 in adult mouse vascular endothelia markedly reduced the vascular leakage.\(^11\) Recombinant Ang1 inhibited both VEGF- and thrombin-induced hyperpermeability in human umbilical vein endothelial cell (HUVEC) monolayers,\(^12\) indicating that Ang1 can directly antagonize the actions of VEGF and thrombin in endothelial cells. These findings raise the possibility that Ang1 has antiinflammatory properties\(^12,13\) in that it prevented the increases in endothelial permeability; however, the signaling pathways responsible for the endothelial barrier protective function have not been elucidated.

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The transient receptor potential canonical (TRPC) subfamily of channel-forming proteins, including TRPC1, are important for regulating Ca\(^{2+}\) entry in endothelial cells\(^{14,15}\) and mediating the increase in endothelial permeability.\(^{16-18}\) Activation of these channels depends on Ca\(^{2+}\) store depletion, hence it is termed capacitative Ca\(^{2+}\) entry (CCE) or store-operated Ca\(^{2+}\) entry.\(^{4,5}\) Studies showed that TRPC1-mediated Ca\(^{2+}\) entry contributed significantly to the thrombin-induced increase in endothelial permeability.\(^{16-18}\) In addition, endothelial cells expressing TRPC1 demonstrated cytoskeletal changes associated with increased endothelial permeability in response to CCE activation; these cells did not express either TRPC3 or TRPC6.\(^{19}\)

In the present study, we addressed the possibility that Ang1 interferes with the CCE pathway activated by VEGF, thereby mitigating the VEGF-induced increase in endothelial permeability. We observed that Ang1 inhibited the Ca\(^{2+}\) entry without affecting the release of intracellular Ca\(^{2+}\) stores. Anti-TRPC1 antibody blocked the VEGF-induced CCE and increase in endothelial permeability. TRPC1 overexpression in endothelial cells augmented the VEGF-induced Ca\(^{2+}\) entry, and Ang1 opposed this effect. On the basis of the immunoprecipitation data, the endothelial barrier protective effect of Ang1 was attributed to inhibition of the association of IP\(_3\)R and TRPC1. These results demonstrate that Ang1 opposes the TRPC1-dependent Ca\(^{2+}\) influx induced by VEGF by interfering with the interaction of IP\(_3\)R and TRPC1, and thereby prevents VEGF-induced increase in endothelial permeability.

### Materials and Methods

VEGF\(_{165}\) and Ang1 were generously provided by Dr John Rudge, Regeneron Pharmaceuticals (Tarrytown, NY). We used recombinant BowAng1, which contains 4 Ang1 fibronogen-like domains fused to the human Fc domain which does not interfere with receptor binding or function.\(^{20}\) HBSS, PBS, and trypsin were purchased from GBBCO BRL Life Technologies. FBS was obtained from HyClone, and EGM-2 was purchased from Clonetics. Transwell chamber inserts were obtained from Corning Costar. Fura-2-AM and BAPTA-AM were obtained from Molecular Probes. Protein A/G-agarose beads were obtained from Santa Cruz Biotechnology.\(^{21}\) BSA tracer was purchased from ICN. Rabbit polyclonal anti-IP\(_3\)R antibody receptor antibody was obtained from Calbiochem. Rat monoclonal anti-VEGFR2 antibody was purchased from R&D Systems. All other reagents, including rabbit polyclonal TRPC1 antibodies, were purchased from Sigma.

### Endothelial Cell Culture and Transfection

Primary HUVECs obtained from Clontech (Palo Alto, Calif) were grown in EGM-2 supplemented with 10% FBS, and passages 3 to 5 were used for all studies. We overexpressed TRPC1 in human dermal microvascular endothelial cells (HMEC-1) obtained from the CDC, because a high transfection efficiency could be achieved in this cell line using described methods.\(^{16}\)

### Transendothelial \(^{125}\)I-Albumin Permeability

Costar Transwell units were used to determine the permeability of \(^{125}\)I-albumin across endothelial cell monolayers.\(^{22}\) Transendothelial clearance rate of \(^{125}\)I-albumin was calculated from: \(CL_i(\muL/min) = \frac{activity_{oc}(cpm/\muL) \times volume_{oc} (\muL)}{activity_{ic}(cpm/\muL) \times time(min)}\), in which \(CL_i\) is clearance (volume of luminal chamber fluid cleared of tracer), \(activity_{oc}\) is counts per \(\muL\) of abluminal chamber sampling, \(volume_{oc}\) is total abluminal chamber volume at the time of sampling, and \(activity_{ic}\) is counts per \(\muL\) of luminal chamber fluid added at the beginning of the experiment.

### Fura-2AM \([\text{Ca}^{2+}]_i\), Measurement

Free cytosolic Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_i\), was measured using fura-2AM as described.\(^{23}\) For the \([\text{Ca}^{2+}]_i\) depletion-repletion protocol, endothelial cells were preloaded with fura-2AM in normal Ca\(^{2+}\)-containing media (1.3 mmol/L) and were placed in Ca\(^{2+}\)-free media immediately before recording; extracellular Ca\(^{2+}\) (1.3 mmol/L) was readded to the media at 300 sec.

### IP\(_3\) Production

Cytosolic IP\(_3\) concentrations were determined with the IP, Biotrak radioimmunoassay system kit using the standard curve according to instructions from Amersham Pharmacia Biotech. HUVECs grown to confluence on 60-mm culture dishes were washed 3 x with PBS and stimulated with 1 \(\muL\) f/ml VEGF for 15 sec, and the reaction was stopped by the addition of ice-cold 15% (v/v) trichloroacetic acid. The scraped endothelial cells were centrifuged for 15 minutes 2000g, and supernatant was washed 3 x with 1 mL water-saturated diethyl ether before neutralization to pH 7.5 using NaHCO\(_3\). Total protein was determined using the DC protein assay following the instructions from Bio-Rad Laboratories.

### Patch Clamp Experiments

Current recordings were made in the whole-cell configuration at a holding potential of \(-50\) mV using an Axopatch 200B amplifier with pClamp 8.1 software and a Digidata 1322 A/D converter (Axon Instruments).\(^{21}\) The extracellular solution contained (in mmol/L): 135 sodium glutamate, 1 MgCl\(_2\), 4 CaCl\(_2\), 10 glucose, and 10 HEPES, pH 7.4 (using NaOH). The pipette solution contained (in mmol/L): 135 sodium glutamate, 10 CsCl, 1 BAPTA, 1 MgCl\(_2\), 1 ATP, 10 HEPES, pH 7.2 (using CsOH). For certain experiments, we used 30 \(\muL\)/0.25-dimethyl-2,5-di(tert-butyl)hydroquinone (BHQ) and 10 mmol/L nonmetabolizable IP\(_3\) (F-IP3).

### Immunoprecipitation and Western Blotting

Western blotting for PLC was done as described.\(^{23}\) Immunoprecipitation and immunoblotting with anti-IP3 receptor and anti-TRPC1 antibodies were performed as previously described.\(^{18}\)

### Statistical Analysis

Two-tailed Student \(t\)-test and one-way ANOVA with Bonferroni post-hoc test were used for statistical comparisons. Values are reported as mean \pm SEM. Differences were considered significant at \(P<0.05\).

### Results

#### Ang1 Inhibits VEGF-Induced Increases in \([\text{Ca}^{2+}]_i\), and Endothelial Permeability

VEGF produced an initial \([\text{Ca}^{2+}]_i\) transient peak followed by a sustained \([\text{Ca}^{2+}]_i\), plateau below the maximum level (Figure 1A). Coadministration of Ang1 inhibited these VEGF-induced \([\text{Ca}^{2+}]_i\), changes in a concentration-dependent manner (Figure 1A and 1B). Ang1 also prevented the VEGF-induced increase in transendothelial \(^{125}\)I-albumin permeability in a similar concentration-dependent manner (Figure 1C).

#### Ang1 Opposes VEGF-Induced Increase in Endothelial Permeability Independent of PLC Activation

Pretreatment of HUVECs with anti-VEGFR2 antibody, U-73122 (PLC inhibitor), or BAPTA-AM (intracellular Ca\(^{2+}\) chelator) prevented the VEGF-induced increases in \([\text{Ca}^{2+}]_i\), and endothelial permeability (Figure 2A through 2D); the inhibition was similar to that seen with Ang1. However, chelerythrine chloride (pan-PKC inhibitor) failed to prevent either response (Figure 2A through 2D). These results dem-
onstrate the importance of PLCγ-dependent Ca²⁺ signaling in the mechanism of VEGF-induced increase in endothelial permeability. We also observed that Ang1 failed to prevent VEGF-activated PLCγ phosphorylation and IP₃ production (Figure 2E and 2F), indicating that Ang1 inhibited the VEGF-mediated increase in endothelial permeability independent of PLCγ activation.

Ang1-Induced Blockade of Plasmalemmal Ca²⁺ Entry Prevents the Increase in Endothelial Permeability

Because VEGF activates intracellular Ca²⁺ store release and extracellular Ca²⁺ influx under normal Ca²⁺ conditions, we used the Ca²⁺ depletion-repletion protocol to address whether Ang1 inhibits intracellular Ca²⁺ store release or plasmalemmal CCE. Addition of VEGF in nominally Ca²⁺-free medium resulted in an increase in [Ca²⁺], attributable to store release alone. The sustained elevation of [Ca²⁺], resulting from CCE was absent until the re-addition of extracellular Ca²⁺ (1.3 mmol/L [Ca²⁺]). In the absence of VEGF exposure, replenishment of [Ca²⁺] had no significant effect on the baseline [Ca²⁺]. Interestingly, the magnitude of CCE was decreased when Ang1 was coadministered with VEGF, whereas the initial peak caused by store release was unaffected (Figure 3A). The magnitude of CCE was reduced even when Ang1 was administered at the time of [Ca²⁺] re-addition (Figure 3B and 3C). Using the same Ca²⁺ depletion-repletion approach, we observed that Ang1 also inhibited the VEGF-induced endothelial monolayer permeability to ¹²⁵I-albumin regardless of whether Ang1 was coadministered with VEGF or added at the time of [Ca²⁺], repletion (Figure 3D).

Ang1 Dampens VEGF- and Direct IP₃-Activated Inward Ca²⁺ Current

We addressed the effect of Ang1 in inhibiting Ca²⁺ influx by patch clamping in single endothelial cells. We activated the lanthanum-sensitive inward current by either extracellular VEGF administration or direct intracellular application of IP₃ via the patch pipette. Ang1 was added to the medium after the VEGF- or IP₃-induced inward current had reached a steady state. We observed that perfusion of Ang1 in the bath solution resulted in significant reductions in both VEGF- and IP₃-induced inward currents (Figure 4A through 4F).

Anti-TRPC1 Antibody Inhibits VEGF-Induced Increases in [Ca²⁺] and Endothelial Permeability

Because TRPC1 channel mediates store-operated Ca²⁺ entry in human endothelial cells,¹⁸ we next addressed the involvement of TRPC1 in the mechanism of VEGF-induced increase in endothelial permeability. We used the antibody that binds amino acids 557 to 571 on the S5 pore-forming region of TRPC1 on the extracellular domain and thereby inhibits Ca²⁺ entry via these channels.¹⁷,²³ Pretreatment of HUVECs for 30 minutes with anti-TRPC1 antibody significantly reduced the sustained phase of Ca²⁺ entry stimulated by VEGF, such that there was no further inhibition with Ang1 (Figure 5A and 5B). The VEGF-induced increase in endothelial permeability was also inhibited by the anti-TRPC1 antibody, and Ang1 produced no further inhibition (Figure 5C).

Ang1 Counters the Augmentation in VEGF-Induced Ca²⁺ Entry Induced by TRPC1 Overexpression

To address whether Ang1 can interfere with TRPC1 function, we determined the effects of Ang1 in modifying the CCE pathway using endothelial cells (HMECs) in which TRPC1 was overexpressed 3-fold.¹⁶ On VEGF administration to
HMECs transfected with the empty vector, we observed the characteristic Ca\(^{2+}\) transient in nominally Ca\(^{2+}\)-free media and the sustained Ca\(^{2+}\) entry on replenishment of [Ca\(^{2+}\)]\(_o\). Coadministration of Ang1 with VEGF in these cells caused a reduction in Ca\(^{2+}\) entry (Figure 6A) as described for HUVECs (Figure 3A and 3B). In the TRPC-1-overexpressing HMECs, we observed augmentation of the Ca\(^{2+}\) entry with VEGF challenge, and the coadministration of Ang1 blocked this effect (Figure 6B and 6C).

**Ang1 Inhibits VEGF-Activated Association of IP\(_3\) Receptor with TRPC1**

As the interaction between IP\(_3\)R and TRPC1 activates store-dependent Ca\(^{2+}\) entry,\(^{18,24}\) we performed immunoprecipitation studies using anti-IP\(_3\)R and anti-TRPC1 antibodies to address the possibility that Ang1 interfered with the association of IP\(_3\)R with TRPC1. We observed that in HUVEC lysates, VEGF increased the association of IP\(_3\)R and TRPC1, and this effect was blocked by the coadministration of Ang1 (Figure 7A and 7B).
We addressed the possible mechanisms by which Ang1 opposes the VEGF-induced increase in endothelial permeability. Based on previous studies suggesting that VEGF increases permeability through a PLC- and Ca\(^{2+}\)/H\(_{11001}\)-dependent pathway,\(^5\)–\(^7\) we have focused on the role of Ang1 in modulating Ca\(^{2+}\)/H\(_{11001}\) entry, and thereby protecting the endothelial barrier. We demonstrated that VEGF increased endothelial permeability through a mechanism involving the PLC-IP\(_3\) pathway and activation of TRPC1-dependent plasmalemmal Ca\(^{2+}\)/H\(_{11001}\) entry. Ang1 inhibited this signaling pathway by opposing the VEGF-induced coupling of TRPC1 and IP\(_3\) without affecting upstream PLC phosphorylation, IP\(_3\) production, or Ca\(^{2+}\) release from intracellular stores. Thus, the results demonstrate that Ang1 blocked the TRPC1-dependent Ca\(^{2+}\) influx induced by VEGF by interfering with the interaction of TRPC1 with IP\(_3\), and thereby abrogated the increase in endothelial permeability.

We used various inhibitors to show that the VEGF-induced permeability response required the PLC-dependent Ca\(^{2+}\) signaling pathway. Our data showed that the endothelial barrier protective effect of Ang1 could not be ascribed to the inhibition of VEGF-induced PLC\(_\gamma\) phosphorylation or IP\(_3\) production. This led us to investigate the downstream components of the PLC pathway, that is, the IP\(_3\)-dependent store release and plasmallemal Ca\(^{2+}\) entry. We observed the complete inhibition of the [Ca\(^{2+}\)] \(_{i}\) transient by Ang1 when Ca\(^{2+}\) release and Ca\(^{2+}\) influx were activated in the presence of normal extracellular Ca\(^{2+}\) concentration. Under these conditions, the [Ca\(^{2+}\)] \(_{i}\) transient is more complex because there is simultaneous activation of intracellular Ca\(^{2+}\) store release and extracellular Ca\(^{2+}\) influx, both of which are coupled to the interaction between IP\(_3\)R and TRPC channels.\(^18\)–\(^25\),\(^26\) We also performed whole-cell patch clamp experiments to assess directly the effects of Ang1 on the inward Ca\(^{2+}\) current. In these experiments, when VEGF was used to elicit the Ca\(^{2+}\) influx and then Ang1 was added, we observed that Ang1 inhibited the lanthanum-sensitive VEGF-induced inward current. These results were consistent with the fura-2AM measurements in which VEGF was first administered and then Ang1 was added, and in which we observed that Ang1 prevented the Ca\(^{2+}\) influx.
We made several observations that support the concept that a threshold [Ca\(^{2+}\)] \(i\) as regulated by the Ca\(^{2+}\) entry is an essential requirement for increasing endothelial permeability. We observed that Ang1 modestly reduced the baseline Ca\(^{2+}\) influx in endothelial cells bathed in normal Ca\(^{2+}\)-containing media, but not when bathed in nominally Ca\(^{2+}\)-free medium. Although in our studies the basal Ca\(^{2+}\) entry was low to begin with, such an effect of Ang1 could explain its action in reducing baseline permeability observed in some studies.12 We also observed that low concentrations of VEGF elicited small Ca\(^{2+}\) transients, whereas higher concentrations were needed to trigger the increase in albumin permeability (unpublished observation). That a minimal threshold of Ca\(^{2+}\) entry is needed to signal the increase in endothelial permeability was also supported by both the Ang1 concentration-dependent responses and Ca\(^{2+}\) depletion-repletion studies. The inhibition of this threshold Ca\(^{2+}\) entry by Ang1 was sufficient to prevent the VEGF-induced increase in endothelial permeability.

TRPC channels are considered good candidates for Ca\(^{2+}\) entry pathways such as CCE. Based on the importance of TRPC1 in regulating the Ca\(^{2+}\) entry-dependent increase in permeability of the human endothelial carrier,16–18 we addressed the role of TRPC1 in the mechanism of Ang1-induced permeability modulation. We demonstrated through overexpression or inhibition of TRPC1 as well as by studying TRPC1 interaction with IP\(_3\)R, that TRPC1 is the likely channel at which Ang1 interferes with the VEGF response. We observed that Ang1 blocked the VEGF-induced interaction of TRPC1 and IP\(_3\). Our data are consistent with the coupling model of CCE whereby activation of PLC\(_{\gamma}\) and generation of IP\(_3\); and the resultant TRPC1-IP\(_3\)R interaction is required for the activation of Ca\(^{2+}\) influx.24–27 It is possible that PLC\(_{\gamma}\) can activate CCE independent of its catalytic function, perhaps by facilitating the localization of the essential components of the Ca\(^{2+}\) entry pathway at the plasma membrane.28 However, the present results do not support this notion as an explanation of the Ang1 effect because we observed that perfusion of Ang1 in the bath solution resulted in a significant reduction in the IP\(_3\)-activated inward current.

Although the present study has focused on Ang1 regulation of TRPC1 activation, we cannot rule out the involvement of other relevant TRPCs. TRPC4 acts as a functional homologue in mouse endothelia to TRPC1 in humans.14,29 For agonist-induced Ca\(^{2+}\) entry in mouse aortic endothelial cells, TRPC4 was essential as either a channel-forming subunit or a constituent required for channel activation.30 Because TRPC1 and TRPC4 can oligomerize,31 it is possible that both may be needed for the VEGF-induced Ca\(^{2+}\) entry. The importance of TRPC4 in regulation of endothelial permeability in mice is reinforced by our observations that the effects of Ang1 on VEGF-induced Ca\(^{2+}\) entry and permeability are mimicked by deletion of the TRPC4 gene in mice (unpublished data). It is also possible that the VEGF-induced activation of Ca\(^{2+}\) entry in

![Figure 4. Ang1 inhibits the inward Ca\(^{2+}\) current induced by VEGF and intracellular application of IP\(_3\).](http://circres.ahajournals.org/doi/figure/10.1161/CIRCRESAHA.117.313949)
can occur via other members of the TRPC family such as TRPC6 which is activated by PLC-generated DAG. However, our data indicate that CCE is required for the VEGF-induced increase in endothelial permeability, and thus it is unlikely that TRPC6, which is not a CCE channel, is involved in the VEGF response.

In summary, the present study demonstrates that Ang1 inhibition of TRPC1-dependent Ca$^{2+}$ influx immediately blocked the VEGF-induced increase in endothelial permeability. The endothelial barrier protective effect of Ang1, mediated by preventing the interaction of TRPC1 with IP$_3$R...
and the resultant inhibition of CCE, suggests a novel therapeutic approach for inflammatory diseases associated with increased microvascular permeability.

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