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Angiopoietin-1 Requires p190 RhoGAP to Protect against Vascular Leakage in Vivo

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Angiopoietin-1 (Ang-1), a ligand of the endothelium-specific receptor Tie-2, inhibits permeability in the mature vasculature, but the mechanism remains unknown. Here we show that Ang-1 signals Rho family GTPases to organize the cytoskeleton into a junction-fortifying arrangement that enhances the permeability barrier function of the endothelium. Ang-1 phosphorylates Tie-2 and its downstream effector phosphatidylinositol 3-kinase. This induces activation of one endogenous GTPase, Rac1, and inhibition of another, RhoA. Loss of either part of this dual effect abrogates the cytoskeletal and anti-permeability actions of Ang-1, suggesting that coordinated GTPase regulation is necessary for the vessel-sealing effects of Ang-1. p190 RhoGAP, a GTPase regulatory protein, provides this coordinating function as it is phosphorylated by Ang-1 treatment, requires Rac1 activation, and is necessary for RhoA inhibition. Ang-1 prevents the cytoskeletal and pro-permeability effects of endotoxin but requires p190 RhoGAP to do so. Treatment with p190 RhoGAP small interfering RNA completely abolishes the ability of Ang-1 to rescue endotoxemia-induced pulmonary vascular leak and inflammation in mice. We conclude that Ang-1 prevents vascular permeability by regulating the endothelial cytoskeleton through coordinated and opposite effects on the Rho GTPases Rac1 and RhoA. By linking Rac1 activation and RhoA inhibition, p190 RhoGAP is critical to the protective effects of Ang-1 against endotoxin. These results provide mechanistic evidence that targeting the endothelium through Tie-2 may offer specific therapeutic strategies in life-threatening endotoxemic conditions such as sepsis and acute respiratory distress syndrome.

Angiopoietin-1 (Ang-1) is a 498-amino acid secreted glycoprotein whose germ line deletion leads to several cardiovascular defects that result in embryonic lethality (1, 2). Although made by numerous cell types, the actions of Ang-1 are primarily mediated by a receptor tyrosine kinase, Tie-2, whose expression is largely restricted to endothelial cells (ECs). Critical roles for Ang-1 and Tie-2 have been described in the formation of the primitive cardiac tube and embryonic vasculature (3). Although necessary for developmental angiogenesis to occur, Ang-1 expression and Tie-2 phosphorylation persist into adulthood in organs not considered angiogenically active (4), suggesting a nonangiogenic role in the mature vasculature. In fact, Ang-1 has been shown to protect adult blood vessels against plasma leakage because of vascular endothelial growth factor or mustard oil (5, 6). However, the mechanism by which Ang-1 defends against vascular leakage in vivo has remained largely unknown.

Permeability is a tightly regulated feature of all vascular beds. A systemic increase in permeability, leading to a degree of vascular leak that impairs organ function, is a hallmark of sepsis, a lethal syndrome of multiorgan dysfunction that arises as a result of disseminated infection. We have previously shown that Tie-2 inhibition induces changes in the endothelial cytoskeleton that are mediated by the small GTPase RhoA and lead to increased cell contraction and enhanced vascular permeability (7). These experiments suggest that the anti-permeability effect of activated Tie-2 may depend on endothelial cytoskeletal forces and cell architecture.

Activated Tie-2 signals through PI3K (8). In turn, PI3K targets numerous effectors, including Akt/PKB, phospholipases, and guanine-nucleotide exchange factors that activate Rho GTPases (9, 10). Two members of the Rho family, RhoA and Rac1, have opposite effects on cells, the former induces actomyosin filament contraction that promotes actin stress fibers that increase centripetal tension throughout the cytoskeleton, and the latter counterbalances this by maintaining adherens and tight junctions between neighboring endothelial cells (11). Rac1 and RhoA are known to mediate opposing changes in EC permeability (the former increasing barrier function and the latter reducing barrier function) induced by a number of ligands (12). Endotoxin increases vascular permeability by activating Rac1 (13, 14). Rho activity is regulated, in part, by the

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inhibitory GTPase activating protein, p190 RhoGAP (15). Rac1 down-regulates RhoA activity through p190 RhoGAP in HeLa cells (16).

Given the known pro-permeability effect of Rho family proteins, the well-described antagonistic relationship between RhoA and Rac1 in the endothelium, and our earlier findings linking Ang-2 to RhoA activation, we hypothesized that Ang-1 may activate Rac1 to achieve its anti-permeability effect. We utilized in vitro assays to elucidate a novel signaling pathway connecting Ang-1 to Rac1 and RhoA, to characterize Ang-1-mediated endothelial cytoskeletal changes, and to test the role of Rho family proteins in the Ang-1-mediated anti-permeability effect against endotoxin. Finally, we used in vivo siRNA to assess the importance of p190 RhoGAP in the pulmonary permeability response to Ang-1 and endotoxin.

**EXPERIMENTAL PROCEDURES**

**Animals**—FVB adult mice (20–25 g) were used in accordance with an IACUC-approved protocol.

**Chemicals**—Human recombinant Ang-1, CD14, and LPS-binding protein were purchased from R&D Systems (Minneapolis, MN). LY294002 is from Cell Signaling Technology (Beverly, MA). Other reagents were obtained from Sigma.

**Cell Culture**—Human microvascular endothelial cells from lung (HMVEC-L) (Cambrex BioScience Walkersville, Inc., Walkersville, MD) were cultured in EBM-2 (Cambrex) supplemented with 5% fetal bovine serum (FBS) and growth factors according to the manufacturer’s instructions. All stimulation experiments were performed after serum starvation with 0.25% FBS/EBM-2 for 24 h. For endotoxin signaling experiments, we used LPS O111:B4 (100 ng/ml), CD14 (100 ng/ml), and LBP (10 ng/ml).

**Western Blot Analysis**—HMVEC-L were lysed in ice-cold RIPA buffer supplemented with protease inhibitors, 1 mmol/liter NaF, and 1 mmol/liter Na3VO4 and prepared for Western analysis as described before (7). Primary antibodies were anti-phospho-Akt Ab (Ser-473), anti-Akt Ab, anti-phosphomoyosin light chain 2 (Ser-19) Ab (Cell Signaling Technology), and anti-glyceraldehyde-3-phosphate dehydrogenase Ab (Chemicon, Temecula, CA). Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and SuperSignal West Dura (Pierce) chemiluminescence substrates were used to detect primary Abs. All Western blots were performed in quadruplicate unless otherwise noted. Representative examples are shown. Densitometry (Image), National Institutes of Health) was used to compare results with unpaired t test.

**Immunoprecipitation**—200 μg of total protein from HMVEC-L lysed in Triton buffer were incubated with anti-Tie-2 Ab (clone Ab33, Upstate Biotechnology, Inc., Lake Placid, NY) or p190 RhoGAP Ab (BD Transduction Laboratories) for 3 h, followed by incubation with protein A-Sepharose (Zymed Laboratories Inc.) for 2 h at 4 °C. After washing the beads, proteins were eluted by heating in SDS sample buffer and detected by immunoblotting with anti-phosphotyrosine (clone 4G10, Upstate Biotechnology, Inc.), anti-Tie-2 Ab, or anti-p190 RhoGAP Ab.

**PI3K Activity Assay**—After signal starvation, HMVEC-L were treated with vehicle or Ang-1 (100 ng/ml) for 15 min.

![FIGURE 1. Ang-1 has opposite effects on Rac1 and RhoA through p190 RhoGAP. A, Ang-1 activates Rac1 and inhibits RhoA. HMVEC-L were incubated with Ang-1, and cells were lysed at the indicated times. GTP-bound active form of Rac1 was collected by pulldown assay and detected by immunoblotting with anti-Rac antibody. Similarly, the GTP-bound active form of RhoA was collected by pulldown assay and detected by immunoblotting with anti-Rho antibody. B, PI3K inhibition blocks Ang-1-induced Rac1 activation. HMVEC-L were incubated with Ang-1 with or without PI3K inhibitor LY294002 (10 μM) for 30 min. GTP-bound active Rac1 was detected as described above. C, active Rac1 is necessary for Ang-1 to inhibit RhoA. Lentiviral delivery of dominant negative p190 RhoGAP was performed as described under “Experimental Procedures.” HMVEC-L were incubated with Ang-1 and Rac and Rho activity were measured as described above. D, Ang-1 induces phosphorylation of p190 RhoGAP in a Rac1-dependent fashion. p190 RhoGAP phosphorylation was detected in vehicle- (Cont) and Ang-1 (100 ng/ml)-treated HMVEC-L as described under “Experimental Procedures” (left panel). HMVEC-L transfected with Rac1T17N lentivirus were treated with control (Cont) or Ang-1 (100 ng/ml) for 30 min (right panel). IP, immunoprecipitation; IB, immunoblot. E, p190 RhoGAP is not required for Ang-1-mediated Rac1 activation but is necessary for Ang-1 to suppress RhoA. p190 RhoGAP knockdown does not block Ang-1-induced Rac1 activation but does block Ang-1-induced RhoA inactivation. siRNA against p190 RhoGAP was transfected as described under “Experimental Procedures.” Panel a, p190 RhoGAP expression in HMVEC-L transfected with siRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is blotted as a loading control. Panel b, HMVEC-L were incubated with vehicle (control) or Ang-1 for 30 min and GTP-bound Rac1 and RhoA were measured as described above. Note: n = 4 per experiment.](https://www.jbc.org/content/282/33/23911/F1)
glutathione S-transferase fusion protein composed of Rac1 or RhoA effector proteins coupled to agarose beads. After washing with lysis buffer, samples were subjected to immunoblotting and detected with anti-Rac1 or anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunocytochemistry**—This assay was conducted as previously described (7) (see Supplemental Material, Methods).

**siRNA Transfection**—Control small interfering RNA (siRNA) (Ambion, Austin, TX) or siRNA directed to human p190 RhoGAP (5’-GGAUUGUGGGAUGU-AAG-3’ and 5’-CUUACAUUCCACAAUCCC-3’) was transfected using SilentFect Lipid reagent (Bio-Rad) according to the manufacturer’s instruction. The cells were used for each experiment 3 days after transfection. Transfection efficiency was 90–100%. Down-regulation of p190 RhoGAP was verified by immunoblotting. We tested two different siRNAs and obtained similar results.

**Lentivirus Construction and Induction**—The dominant negative form of Rac1 (Rac1T17N) and the constitutively active form of RhoA (RhoAG14V) were constructed by PCR using pcDNA-Rac1T17N or -RhoAG14V (University of Missouri, Rolla, cDNA Resource Center) as a template and subcloned into the pHAGE lentiviral backbone vector at the NotI/BamHI sites. Generation of lentiviral vectors was accomplished by a five-plasmid transfection procedure (17) (see Supplemental Material, Methods).

**In Vivo Permeability Assay**—Mice (8–12 weeks old, female FVB strain) were pretreated with Ang-1 (10 μg, intraperitoneally). 8 h after the first Ang-1 injection, the second dose of Ang-1 (10 μg, intraperitoneally) and LPS (100 μg, intraperitoneally) were co-injected. Lung permeability was assessed 16 h after the second injection as described previously (7) (see Supplemental Material, Methods).

**Histology**—Lungs were harvested, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**In Vivo Delivery of siRNA**—Delivery of siRNA into mice was performed using TransIT® hydrodynamic delivery solution (Mirus, Madison, WI) per the manufacturer’s instructions. Mice were injected with either 10 μg of control siRNA or 10 μg of p190 RhoGAP siRNA in 2 ml of delivery solution injected into the tail vein over 7 s. The method of siRNA delivery is...
p190 RhoGAP Is Necessary for Ang-1 Anti-permeability

Ang-1 activates Rac1, but it did not suppress RhoA activity when ECs were infected with a dominant negative form of Rac1 (Rac1T17N) using a lentiviral vector (Fig. 1C). Rac1T17N also reduced Ang-1-induced p190 RhoGAP activity by 60% (p < 0.05, Fig. 1D). Suppression of p190 RhoGAP by siRNA (Fig. 1E, panel a) did not affect Ang-1-induced Rac1 activation (Fig. 1E, panel b, upper panel), but it did abolish the inhibitory effect of Ang-1 on RhoA (Fig. 1E, panel b, lower panel); this response was analogous to the effect of Ang-1 on RhoA in the setting of dominant negative Rac1 (Fig. 1C). These results show that Ang-1 activates endothelial Rac1 and p190 RhoGAP to inhibit RhoA and that p190 RhoGAP acts downstream of activated Rac1.

Enhancement of Endothelial Junctions by Ang-1 Requires PI3K and Rac1, Not p190 RhoGAP—Centripetal force exerted on the actin cytoskeleton can increase EC permeability. This is resisted by homologous binding between VE-cadherins, the transmembrane proteins that maintain adherens junctions between neighboring ECs. The cytoskeletal distribution of actin and myosin distinguishes these states: central fibers reflect centripetal force, whereas peripheral fibers indicate junctional preservation (20). These fibers arise from actin–myosin cross-bridges that are stimulated by myosin light chain phosphorylation (MLC-p) (20). RhoA regulates MLC-p through Rho-associated kinase (21, 22) inducing central stress fibers that pull cell borders centripetally, whereas Rac1 augments junctional VE-cadherin and induces peripheral fibers.

Ang-1 induces MLC-p, peaking at 0.5–1.0 h after stimulation (supplemental D). By immunofluorescence microscopy, we observed an increase in cortical actin, peripheral MLC-p, and junctional VE-cadherin compared with control conflu-
gaps with attenuated VE-cadherin staining (Fig. 2A, panels i–l).

We next studied the effect of dominant negative Rac1 (T17N) and constitutively active RhoA (RhoAG14V) on endothelial architecture. Lentiviral delivery of Rac1T17N or RhoAG14V produced intercellular gaps (Fig. 2B, panels b and c) that were not present in control virus-infected cells (Fig. 2B, panel a). In the presence of control virus, Ang-1 retained the ability to augment junctional VE-cadherin staining (Fig. 2B, panel d). However, this effect of Ang-1 was markedly diminished in Rac1T17N- and RhoAG14V-treated cells, resulting in gap formation (Fig. 2B, panels e and f). These results show that inhibition of endogenous Rac1 or activation of RhoA is sufficient to prevent Ang-1-mediated junctional fortification.

Knockdown of p190 RhoGAP siRNA did not attenuate junctional VE-cadherin staining nor did it promote intercellular gap formation (supplemental Fa). Moreover, Ang-1 retained its ability to increase junctional VE-cadherin staining despite p190 RhoGAP knockdown (supplemental Fb), suggesting that p190 RhoGAP is dispensable for Ang-1 induced structural effects in otherwise unstimulated cells.

p190 RhoGAP Is Necessary for Ang-1 to Block Endotoxin-induced Structural Disruption—Endotoxin treatment (100 ng/ml) of ECs decreased Rac1 activity by 25% (p < 0.01) and induced RhoA activity by 30% (p < 0.01; Fig. 3A). Both of these effects were reversed by co-incubation with Ang-1 (p < 0.01; Fig. 3A). When endogenous p190 RhoGAP expression was blocked, Ang-1 could no longer suppress endotoxin-mediated RhoA activation (p < 0.05; Fig. 3B). Using immunofluorescence microscopy, we observed that 30 min of endotoxin exposure (100 ng/ml) scattered and attenuated junctional VE-cadherin staining and promoted gap formation compared with vehicle-treated cells (Fig. 3C, panels a and b). Ang-1 prevented these effects, and cell-cell junctions appeared normal (Fig. 3C, panel c). Rac1T17N (Fig. 3C, panel d) or p190 RhoGAP siRNA (Fig. 3C, panel e) greatly diminished the ability of Ang-1 to rescue endotoxin-treated cells. Control virus or control siRNA had no effect on the response to endotoxin or endotoxin plus Ang-1 (data not shown). These results show that Ang-1 requires p190 RhoGAP to augment junctions weakened by endotoxin-mediated RhoA activation.

Ang-1 Blocks Endotoxin-induced Hyperpermeability through PI3K, Rac1, and p190 RhoGAP—We next tested the effects of Ang-1 and endotoxin on permeability using a standard in vitro assay to quantify the flux of fluorescently labeled albumin across a confluent EC monolayer. Ang-1 did not significantly affect basal permeability; endotoxin increased it by 20%, and the combination restored normal permeability (Fig. 4A). The protective effect of Ang-1 was lost when LY294002 was added (Fig. 4A). In the presence of Rac1T17N, basal permeability was increased, endotoxin did not further augment the trans-monolayer leak, and Ang-1 failed to reverse the hyperpermeability (Fig. 4B). In contrast, p190 RhoGAP siRNA had little effect on basal permeability but did prevent the protective effect of Ang-1 (Fig. 4C).

Inhibition of p190 RhoGAP Abolishes the Protective Effect of Systemic Ang-1 against Endotoxemic Vascular Leak
cle cells), basement membrane, and connective tissue found in whole vessels of living animals.

Evans blue dye avidly binds to serum albumin and therefore can be used as a tracer for macromolecule flux across the microvasculature. In the lung, systemically administered endotoxin \( (100 \mu g, \text{intraperitoneally}) \) produced an 8-fold increase in dye extravasation compared with controls (Fig. 5A). The increased permeability was blocked by Ang-1 (Fig. 5A). Light photomicrographs of lung sections revealed that systemic endotoxin resulted in interstitial edema and leukocyte infiltration as compared with control lung sections and those taken from animals pretreated with Ang-1 (Fig. 5B).

Delivery of p190 RhoGAP siRNA (10 \( \mu g, \text{intravenously} \)) effectively reduced p190 RhoGAP expression in the lung (Fig. 5C, panel a) and was sufficient to block the anti-permeability effect of Ang-1 (Fig. 5C, panel b). Moreover, histological sections confirmed that p190 RhoGAP knockdown abrogated the ability of Ang-1 to block endotoxin-mediated interstitial edema and leukocyte infiltration (Fig. 5D). These results validate our earlier \textit{in vitro} findings in the context of the whole lung in healthy adult rodents and also establish the critical contribution of p190 RhoGAP to Ang-1-mediated protection against vascular leakage.

**DISCUSSION**

The data presented here demonstrate that Ang-1 protects against endotoxin-mediated vascular leakage by preventing cytoskeletal rearrangements in the EC that are normally induced by this bacterial toxin. To achieve this, Ang-1 signals through PI3K to activate Rac1, phosphorylate p190 RhoGAP, and inhibit RhoA activity. Ang-1 is able to block the structural rearrangements and hyperpermeability induced by endotoxin but requires p190 RhoGAP to do so. To demonstrate its importance more conclusively, expression of p190 RhoGAP was inhibited \textit{in vivo} using siRNA. A schematic summarizing the dichotomous actions of Ang-1 and endotoxin on the Rac1/RhoA balance is presented in Fig. 6.

Inhibition of either Rac1 or p190 RhoGAP negates the structural and functional protective effects of Ang-1 against endotoxin. The ability of Rac1 to prevent permeability is well known, but the ability of p190 RhoGAP to attenuate pro-permeability signaling \textit{in vivo} has not yet been described. Although p190 RhoGAP suppression does not alter baseline cytoskeletal structure or \textit{in vitro} permeability (or \textit{in vivo} permeability, data not shown), its expression and activation become crucial when RhoA is independently stimulated. Our results suggest that endotoxin-mediated activation of RhoA can be blocked by simultaneous activation of p190 RhoGAP by Ang-1. This is consistent with the regulatory role described for p190 RhoGAP in other cell types (23). Therefore, although p190 RhoGAP may be dispensable for the endothelium at baseline, it is crucial for the endothelium to prevent the structural rear-

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**FIGURE 5.** Ang-1 blocks LPS-induced pulmonary hyperpermeability \textit{in vivo} in a p190 RhoGAP-dependent fashion. A, lung permeability (measured in absorbance units, see “Experimental Procedures”) was determined in control (Cont) mice (vehicle intraperitoneally), endotoxin (LPS 100 \( \mu g \) intraperitoneally), or endotoxin plus Ang-1 (10 \( \mu g \) intraperitoneally, 2 doses). B, hematoxylin and eosin-stained \( \times 40 \) photomicrographs of lungs taken from animals treated as in A above. LPS results in edema and leukocyte infiltration that are reversed by Ang-1. C, \textit{in vivo} delivery of p190 RhoGAP siRNA. Panel a, p190 RhoGAP protein is reduced in mouse lung after hydrodynamic delivery of specific siRNA but not after delivery of control siRNA. Panel b, in mice treated with control siRNA, endotoxin-induced permeability was unaffected as was the rescue ability of Ang-1; p190 RhoGAP knockdown blocked the anti-permeability effect of Ang-1 \textit{in vivo}. p190 siRNA treatment in the absence of endotoxin caused no change in basal lung permeability compared with control siRNA (data not shown). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. D, hematoxylin and eosin-stained \( \times 40 \) photomicrographs of lungs taken from animals treated as in C above. In the presence of p190 RhoGAP knockdown, Ang-1 can no longer inhibit endotoxin-induced edema and inflammation. *, \( p < 0.05 \). Mean \pm S.E. of four experiments.
rangements induced by RhoA activators that can ultimately compromise barrier function.

Our results show that endogenous Rac1 activity is necessary to maintain cell-cell adhesion and prevent excess permeability. Rac1 may affect intracellular VE-cadherin distribution by stabilizing it at cell junctions through post-translational modification (24, 25). Indeed, our experiments support this assertion as we observed clear attenuation of junctional VE-cadherin staining when Rac1 was inactivated (by chemical inhibition of PI3K or dominant negative Rac1) or when RhoA was activated (by LPS or constitutively active RhoA), yet the total level of VE-cadherin was unchanged across these conditions (data not shown).

VE-cadherin may also activate Rac1 (26) to promote p190 RhoGAP-mediated RhoA suppression, resulting in less centripetal tension and cell contraction (27, 28). If junctional proteins such as VE-cadherin activate Rac1 and Rac1 in turn promotes junctional localization of VE-cadherin, a positive feedback loop may exist to maintain basal barrier function, analogous to that observed in epithelial cell-cell adhesion (29). Our results with dominant negative Rac1 clearly highlight the importance of one limb in this loop, and work by Dejana and co-workers (30) convincingly illustrates the importance of VE-cadherin, as well, for maintenance of barrier function.

In addition to Ang-1 and endotoxin, several other extracellular signals also regulate endothelial permeability through RhoA and/or Rac1, such as thrombin, histamine (31), sphingosine 1-phosphate (32), lysophosphatidic acid (33), transforming growth factor-β (34), and ligands of VCAM-1 and ICAM-1 (35, 36). Therefore, ample evidence suggests that competing Rho GTPases may provide a downstream conserved mechanism for regulation of vascular permeability by controlling EC shape and
adhesion responses to diverse stimuli. Through its action on p190 RhoGAP, Ang-1 may be able to counteract other endothelial RhoA activators as well. Other endothelial PI3K activators may transduce a similar protective effect against permeability.

Rho GTPases mediate other aspects of EC behavior that could contribute to the anti-permeability effect of Ang-1. These include redox signaling through NADPH oxidase (37), secretion of signaling molecules (38), and adhesion of leukocytes (14). Our in vivo results were notable for the ability of Ang-1 to block endotoxin-induced infiltration of leukocytes into the lung parenchyma. Ang-1 down-regulates expression of VCAM-1, ICAM-1, and E-selectin, thereby preventing initial leukocyte adhesion (39, 40). Rolling leukocytes induce clustering of these adhesion proteins that leads to RhoA activation and results in formation of interendothelial gaps through which leukocytes cross the endothelium (41). Therefore, the anti-inflammatory effect of Ang-1 may arise both due to decreased adhesion molecule expression in ECs and due to suppression of clustering-induced RhoA activation. This latter effect may further augment the anti-permeability action of Ang-1 in vivo, by preventing a leukocyte-induced secondary increase in permeability.

To our knowledge, the results presented here are the first direct demonstration of vascular permeability regulation in vivo using siRNA (rather than a chemical inhibitor) for a Rho family protein. Because Ang-1 acts on Tie-2, an endothelial-restricted receptor, we infer that its anti-permeability effect is mediated through the EC. Therefore, the simplest hypothesis to account for the effect of p190 RhoGAP siRNA is that p190 RhoGAP expression within the pulmonary endothelium is the critical transducer of Ang-1 protection against vascular leak in the lung. Other possibilities are less likely but cannot be excluded (such as Ang-1 acting on non-EC types or p190 RhoGAP suppression in another cell type indirectly attenuating the protective effect of Ang-1).

This works extends a prior study that showed Ang-1 overexpression protected mice from endotoxin shock (42). Ang-2, considered a context and dose-dependent Tie-2 antagonist, appears to be required for the acute inflammatory response (43) and for hyperoxia-induced lung injury as has been recently shown with Ang-2 null mice and lung-targeted Ang-2 siRNA (44). In some settings, such as high doses administered in vivo, Ang-2 may actually activate Tie-2 and protect against permeability (45). When considered together, our current findings and these prior studies suggest that Tie-2 activation (by Ang-1 or perhaps high dose Ang-2) may be critical in the defense of the vascular permeability barrier.

Several additional questions are raised by our findings. The critical effectors downstream of Rac1 regulating Ang-1-induced junctional stabilization and anti-permeability need to be enumerated. α-Catenin may bridge the signaling pathway connecting Rac1 to VE-cadherin (46), but this has not been tested in the setting of Ang-1 stimulation. The ability of Ang-1 to suppress RhoA may depend on the ligand and pathway activating RhoA (47). Furthermore, the endothelial role of Cdc42, the third major GTPase, remains to be clarified as one report suggests no effect on endothelial permeability (12), whereas a more recent study suggests that Cdc42 stabilizes junctional proteins (46). Although our in vitro studies have focused on paracellular gap formation as the mechanism for permeability, the relative contribution of transcytotic (e.g. caveolae) pathways, particularly in vivo, remains to be defined in this system. However, recent data do suggest that the ability of Ang-1 to close paracellular gaps in vivo accounts for its anti-permeability action (48). Calcium influx induces permeability downstream of several ligands, most notably thrombin (49). Given recent evidence that Ang-1 blocks vascular endothelial growth factor-induced permeability in vitro by inhibiting calcium influx through TRPC-1 (50), that Ang-1 also appears to activate protein kinase Cζ (51), and that key permeability effector proteins, such as myosin light chain kinase, are downstream of both the RhoA and calcium-calmodulin pathways (reviewed in Ref. 52), it is quite possible that Ang-1 utilizes multiple signaling pathways to control permeability. Finally, the regulation of p190 RhoGAP expression and activation within the endothelium merit further examination, particularly given a recent characterization of its anti-RhoA signaling downstream of another permeability effector, focal adhesion kinase (28).

Our results provide a novel mechanism for the anti-permeability effect of Ang-1 in the vascular system and describe, in detail, competing effects on endothelial cytoskeletal structure and cell-cell adhesion from two GTPase signaling pathways that regulate vascular permeability. These data suggest that activation of endothelial p190 RhoGAP is critical for Ang-1 to block endotoxin-induced vascular leak and inflammation in vivo. Finally, this report affirms the importance of the endothelium in the defense against endotoxemic injury.

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REFERENCES
1. Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonnierp, P. C., and Yancopoulos, G. D. (1996) Cell 87, 1161–1169
2. Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonnierp, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996) Cell 87, 1171–1180
3. Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995) Nature 376, 70–74
4. Wong, A. L., Haraon, Z. A., Werner, S., Dewhirst, M. W., Greenberg, C. S., and Peters, K. G. (1997) Circ. Res. 81, 567–574
5. Thurston, G., Suri, C., Smith, K., McClain, J., Sato, T. N., Yancopoulos, G. D., and Mcdonald, D. M. (1999) Science 286, 2511–2514
6. Thurston, G., Rudge, J. S., Ioffe, E., Zhou, H., Ross, L., Croll, S. D., Glazer, N., Holash, J., McDonald, D. M., and Yancopoulos, G. D. (2000) Nat. Med. 6, 460–463
7. Parikh, S. M., Mammoto, T., Schultz, A., Yuan, H. T., Christiani, D., Karumanchi, S. A., and Sukhatme, V. P. (2006) Plos Med. 3, e46–e60
8. Cho, C. H., Kammerer, R. A., Lee, H. J., Yasunaga, K., Kim, K. T., Choi, H. H., Kim, W., Kim, S. H., Park, S. K., Lee, G. M., and Koh, G. Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 5553–5558
9. Welch, H. C., Coadwell, W. J., Stephens, L. R., and Hawkins, P. T. (2003) FEBS Lett. 546, 93–97
10. Wymann, M. P., and Marone, R. (2005) Curr. Opin. Cell Biol. 17, 141–149
11. Burridge, K., and Wennerberg, K. (2004) Cell 116, 167–179
12. Wojciak-Sothard, B., Potempa, S., Eichholtz, T., and Ridley, A. J. (2001) J. Cell. Sci. 114, 1343–1355
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13. Essler, M., Staddon, J. M., Weber, P. C., and Aeppelbacher, M. (2000) J. Immunol. 164, 6543–6549
14. Thorlacius, K., Slotta, J. E., Laschke, M. W., Wang, Y., Menger, M. D., Jeppsson, B., and Thorlacius, H. (2006) J. Leukocyte Biol. 79, 923–931
15. Settleman, J., Albright, C. F., Foster, L. C., and Weinberg, R. A. (1992) Nature 359, 153–154
16. Nimmual, A. S., Taylor, L. J., and Bar-Sagi, D. (2003) Nat. Cell Biol. 5, 236–241
17. Mostoslavsky, G., Kotton, D. N., Fabian, A. I., Gray, J. T., Lee, J. S., and Mulligan, R. C. (2005) Mol. Ther. 11, 932–940
18. Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A., and Herweijer, H. (2002) Nat. Genet. 32, 107–108
19. Liu, F., Song, Y., and Liu, D. (1999) Gene Ther. 6, 1258–1266
20. Dudek, S. M., and Garcia, J. G. (2004) J. Appl. Physiol. 91, 1487–1500
21. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997) Science 275, 245–248
22. Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997) Science 275, 1308–1311
23. Arthur, W. T., and Burridge, K. (2001) Arterioscler. Thromb. Vasc. Biol. 21, E127–E133
24. Wojciak-Stothard, B., Tsang, L. Y., and Haworth, S. G. (2005) J. Biol. Chem. 280, 33305–33308
25. Seebach, J., Madler, H. J., Wojciak-Stothard, B., and Schnittler, H. J. (2005) Thromb. Haemostasis 94, 620–629
26. Lampugnani, M. G., Zanetti, A., Breviario, F., Balconi, G., Orsenigo, F., Corada, M., Spagnuolo, R., Betson, M., Braga, V., and Dejana, E. (2002) Mol. Biol. Cell 13, 1175–1189
27. Noren, N. K., Niessen, C. M., Gumbiner, B. M., and Burridge, K. (2001) J. Biol. Chem. 276, 33305–33308
28. Holinstat, M., Knezevic, N., Broman, M., Samarel, A. M., Malik, A. B., and Mehta, D. (2006) J. Biol. Chem. 281, 2296–2305
29. Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000) Cell 100, 209–219
30. Corada, M., Mariotti, M., Thurston, G., Smith, K., Kunkel, R., Brockhaus, M., Lampugnani, M. G., Martin-Padura, I., Stoppacciaro, A., Ruco, L., McDonald, D. M., Ward, P. A., and Dejana, E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9815–9820
31. van Nieuw Amerongen, G. P., van Delft, S., Vermeer, M. A., Collard, J. G., and van Hinsbergh, V. W. (2000) Circ. Res. 87, 335–340
32. Garcia, J. G., Liu, F., Verin, A. D., Birulova, A., Dechert, M. A., Gerthoffer, W. T., Bamberg, J. R., and English, D. (2001) J. Clin. Investig. 108, 689–701
33. van Nieuw Amerongen, G. P., Vermeer, M. A., and van Hinsbergh, V. W. (2000) Arterioscler. Thromb. Vasc. Biol. 20, E127–E133
34. Clements, R. T., Minnear, F. L., Singer, H. A., Keller, R. S., and Vincent, P. A. (2005) Am. J. Physiol. 288, L294–L306
35. Laudanna, C., Campbell, J. J., and Butcher, E. C. (1996) Science 271, 981–983
36. Wojciak-Stothard, B., Williams, L., and Ridley, A. J. (1999) J. Cell Biol. 145, 1293–1307
37. Ushio-Fukai, M., Tang, Y., Fukai, T., Dikalov, S. I., Ma, Y., Fujimoto, M., Quinn, M. T., Pagano, P. J., Johnson, C., and Alexander, R. W. (2002) Circ. Res. 91, 1160–1167
38. Ettienne-Manneville, S., and Hall, A. (2002) Nature 420, 629–635
39. Gamble, J. R., Drew, J., Trezise, L., Underwood, A., Parsons, M., Kasmin, L., Rudge, J., Yancopoulos, G., and Vadas, M. A. (2000) Circ. Res. 87, 603–607
40. Kim, I., Moon, S. O., Park, S. K., Chae, S. W., and Koh, G. Y. (2001) Circ. Res. 89, 477–479
41. Millan, J., and Ridley, A. J. (2005) Biochem. J. 385, 329–337
42. Witzenbichler, B., Westermann, D., Knueppel, S., Schultheiss, H. P., and Tschop, C. (2005) Circulation 111, 97–105
43. Fiedler, U., Reiss, Y., Scharpfenecker, M., Grunow, V., Koidl, S., Thurston, G., Gale, N. W., Witzenrath, M., Rosseau, S., Sutter, N., Sobke, A., Herrmann, M., Preissner, K. T., Vajkoczy, P., and Augustin, H. G. (2006) Nat. Med. 12, 235–239
44. Bhandari, V., Choo-Wing, R., Lee, C. G., Zhu, Z., Nedrelow, J. H., Chupp, G. L., Zhang, X., Matthay, M. A., Ware, L. B., Homer, R. J., Lee, P. J., Geick, A., de Fougereolles, A. R., and Elias, J. A. (2006) Nat. Med. 12, 1286–1293
45. Daly, C., Pasnikowski, E., Burova, E., Wong, V., Aldrich, T. H., Griffiths, J., Ioffe, E., Daly, T. J., Fandl, J. P., Papadopoulos, N., McDonald, D. M., Thurston, G., Yancopoulos, G. D., and Rudge, J. S. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 15491–15496
46. Bromer, M. T., Kouklis, P., Gao, X., Ramchandran, R., Neamu, R. F., Minshall, R. D., and Malik, A. B. (2006) Circ. Res. 98, 73–80
47. Pizurki, L., Zhou, Z., Glynos, K., Roussos, C., and Papapetropoulos, A. (2003) Br. J. Pharmacol. 139, 329–336
48. Baffert, F., Le, T., Thurston, G., and McDonald, D. M. (2006) Am. J. Physiol. 290, H107–H118
49. Lum, H., Del Vecchio, P. J., Schneider, A. S., Goligorsky, M. S., and Malik, A. B. (1999) J. Appl. Physiol. 86, 1471–1476
50. Jho, D., Mehta, D., Ahmmed, G., Gao, X. P., Tiruppathi, C., Broman, M., and Malik, A. B. (2005) Circ. Res. 96, 1282–1290
51. Li, X., Hahn, C. N., Parsons, M., Drew, J., Vadas, M. A., and Gamble, J. R. (2004) Blood 104, 1716–1724
52. Wojciak-Stothard, B., and Ridley, A. J. (2002) Vascular Pharmacol. 39, 187–199
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